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Synthetic routes towards spin probe-grafted copolymers

Christopher McCarthy

SYNTHETIC ROUTES TOWARDS SPIN PROBE-GRAFTED COPOLYMERS

CHRISTOPHER J. McCARTHY

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APPROVED:

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Project Adviser

_____

Department Head

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Rochester Institute of Technology
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ABSTRACT

Nitroxide spin labels can be useful as oxygen probes since the shape of the characteristic three line electron spin resonance (ESR) spectrum broadens in the presence of molecular oxygen. In order to use these probes *in vivo* to measure oxygen content of tumors, contact of toxic nitroxides with tissues must be prevented. This study describes synthesis and characterization of several styrene/maleic anhydride copolymers containing grafted 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (TEMPO) spin probes. Infrared (IR) spectroscopy was used to determine the structure of the copolymers. ESR spectroscopy was used to contrast the free radical activity of the copolymer in an nitrogen-rich and oxygen-rich environment. The changes observed in the ESR spectra were that dissolved oxygen noticeably broadens the peaks, but significant free radical activity remains in the copolymers. Attempts to graft TEMPO to 92/8 [styrene/maleic anhydride] copolymers achieved 67% success and to 50/50 [styrene/maleic anhydride] copolymers achieved 40% success.

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SPECTRA

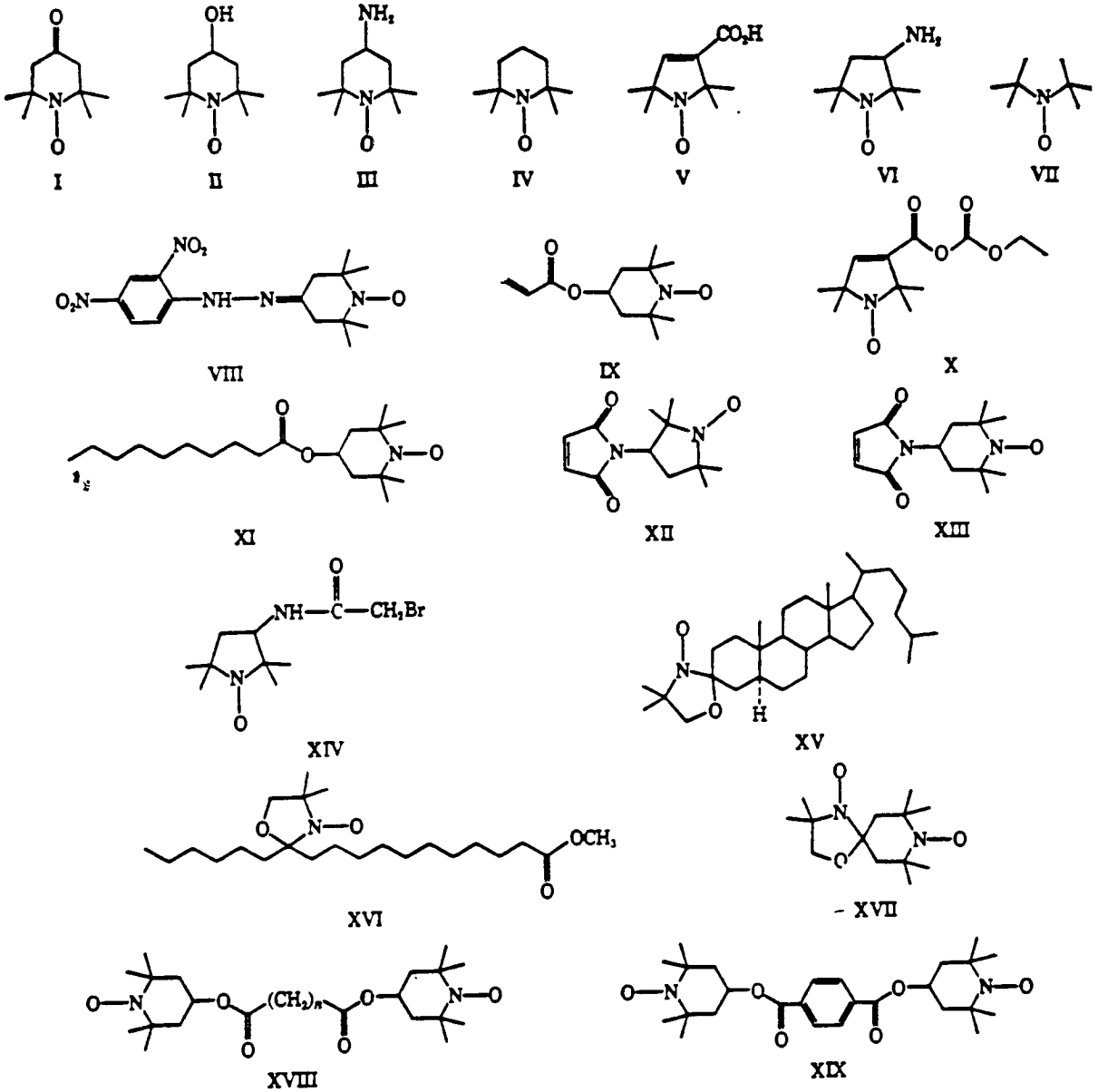
INTRODUCTION

The objective of this project is the synthesis and characterization of nitroxide-grafted free radical copolymers. Because natural occurrence of paramagnetism in biological systems is low, introduction of an external probe containing a free radical can supply important information about intracellular events. These polymers could be useful in monitoring the *in vivo* oxygen content of tumors, since several tumor models and therapy schemes are based on oxygen content of the tissue. Even though molecular oxygen is also paramagnetic, nitroxide free radicals are more easily detected by common electron spin resonance (ESR) instruments. The nitroxide radical displays a characteristic three line ESR spectrum in a relatively nonviscous liquid, where the nitroxide has translational and rotational freedom. The shape of this spectrum is very sensitive to the oxygen concentration of the nitroxide solution, and therefore is a good probe for dissolved oxygen. Unfortunately, nitroxides are relatively toxic and are quickly ingested by living tissues, so they must be shielded from their host. One way of doing this is to encapsulate them in a polymer.

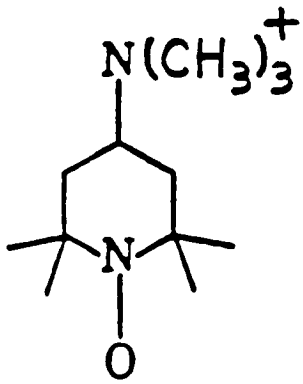
Common nitroxide spin labels are extraordinarily stable and survive synthetic conditions. This is partly due to the delocalization of the free radical over the nitrogen and the oxygen. Most commercially available nitroxides have methyl groups adjacent to the nitroxide to prevent a transfer of the free radical. The methyl groups provide steric

hindrance to further increase the stability of the nitroxide. Some examples of nitroxide compounds are given in Figure 1[1]. Nitroxides I through VI have a reactive functional group used for spin labelling. Compound VIII, for example, was prepared from ketone I. Compounds IX and XI were prepared from the alcohol nitroxide II. Similar reactions can be performed with amines and acids to obtain compounds for different purposes. For instance, a spin probe can be labelled to a steroid, like compound XV, or a fatty ester, like compound XVI. Compounds V, IX, X, XII, and XIII have a carbon-carbon double bond attached to the spin probe which may be utilized for alkylation additions. Compound XVIII is a spin labelled polymer. The ability of the nitroxide to survive synthetic conditions allows it to be linked to a wide variety of compounds.

Figure 1. Some Examples of Nitroxide Molecules[1]



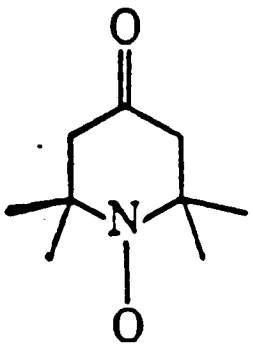
Several nitroxide compounds have been used for oxygen concentration measurements. Chan, Glockner, and Swartz used 2,2,6,6-tetramethyl-piperidine-N-oxyl-4-trimethylammonium (Cat1) in studies of oxygen consumption rates by thymus-bone-marrow cells[2].



Cat1

In order to avoid the reduction of the nitroxide, the positively charged Cat1 was contained in liposomes. The positive charge of Cat1 prevented it from escaping the liposome. ESR spectra of encapsulated Cat1 was found to be nearly as sensitive to the concentration of oxygen as free Cat1. The difference in sensitivity was attributed to the combination of three unavoidable problems. The most significant of which was the rapid reduction of free Cat1 by reducing agents of the bone-marrow cells such as ascorbic acid. Secondly, there was a time lag in the response of the encapsulated spin probe due to the time required for oxygen to diffuse across the liposome membrane. Thirdly, the concentration of Cat1 in the liposome was much higher than the overall solution because the volume occupied by the liposome was about twenty percent of the total volume of the suspension. The higher concentration of Cat1 in the liposome resulted in a greater degree of Heisenberg spin exchange. In spite of

these problems, the agreement of results using these two methods indicates that the liposomal membrane did not significantly hinder nitroxide-oxygen interactions. Another nitroxide compound used in vivo oxygen concentration measurements of the peritoneal cavity of a mouse by Subczynski, Lukiewicz, and Hyde, was 1-oxyl-2,2,6,6-tetramethyl-4-piperidone (TEMPONE)[3].



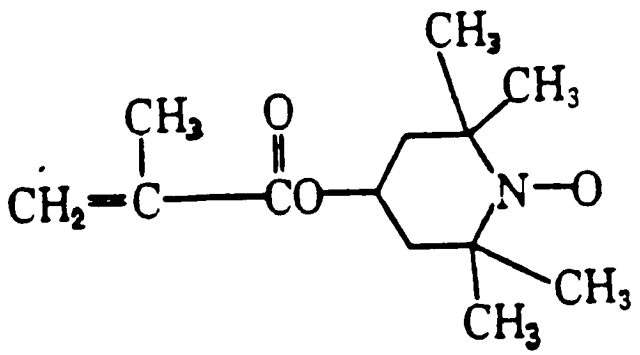
TEMPONE

Because spin labels in the blood stream are rapidly reduced, the nitroxide was contained in a small capsule made from oxygen permeable plastic TPX, a methylpentene polymer. Complications arose from changes in the body temperature and from movement of the capsule caused by respiratory motion. As the mouse breathed different concentrations of oxygen, the concentration of oxygen measured in the peritoneal cavity was similar to the amount that would be measured in water under the same conditions. The distinct advantage of this method of spin-label oximetry is the convenience and speed with which the experiment can be performed. No wires pass through the body and the polymer capsule had no apparent ill effects.

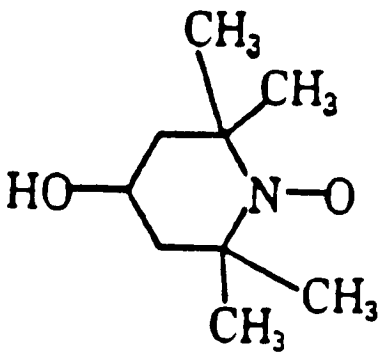
In addition to oximetry, spin labels are useful to determine the

binding properties of compounds. Kunicki and Lai generated TEMPO-maleimide labelled antibodies to monitor integral membrane protein mobility on the surface of the intact cell[4]. Fortunately, the antibodies were able to retain their binding abilities after being spin labelled. As a general rule, the more immobilized nitroxide will display a broader ESR spectra. Binding of the spin labelled antibodies to the surface of the cell was easily detected because its ESR spectra was broadened considerably

Nitroxides polymers have been prepared by various methods. The work of Griffith and Waggoner prepared nitroxide polymers by two different methods[5]. First, a methacrylate ester nitroxide monomer was polymerized by the method of Goode.



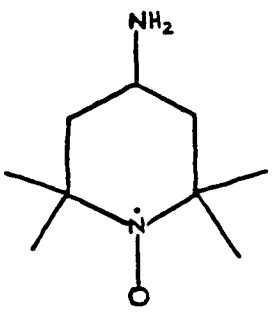
A nitroxide monomer solution was added dropwise to a solution of phenylmagnesium bromide and stirred at room temperature for one hour. The product was of low molecular weight. In the second approach the nitroxide 2,2,6,6-tetramethyl-4-piperidinol-1-oxyl (shown below) was attached to a previously prepared maleic anhydride-methyl vinyl ether (1:1) copolymer.



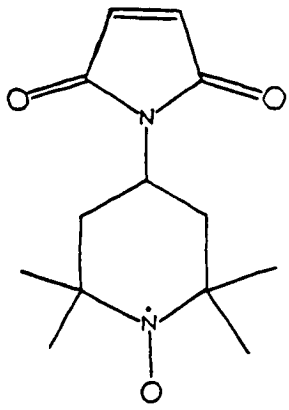
piperidinol

The copolymer and nitroxide were reacted with p-toluenesulfonic acid and hydroquinone at 100 C for five minutes. A nitrogen analysis of this reaction product revealed that approximately one-half of the anhydride groups in the original copolymer had reacted with the nitroxide alcohol to give the corresponding half-ester.

In my research, two synthetic strategies are examined for the making of a nitroxide grafted copolymer. The first is a free radical copolymerization of styrene and 4-maleimido-TEMPO. The second is the grafting of 4-amino-TEMPO to a styrene/maleic anhydride copolymer. The structures of these two nitroxides are shown below.

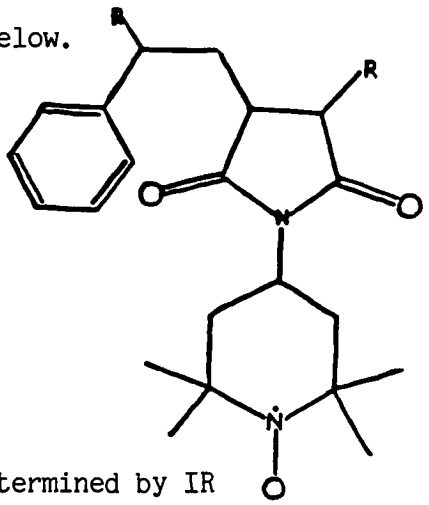


4-amino-TEMPO



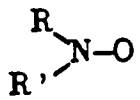
4-maleimido-TEMPO

Although 4-Maleimido-TEMPO is commercially available, it is very expensive. It was therefore synthesized in our laboratories by the procedure of Hamilton and McConnell[6]. Both synthetic strategies may lead to a copolymer with the general structure shown below.

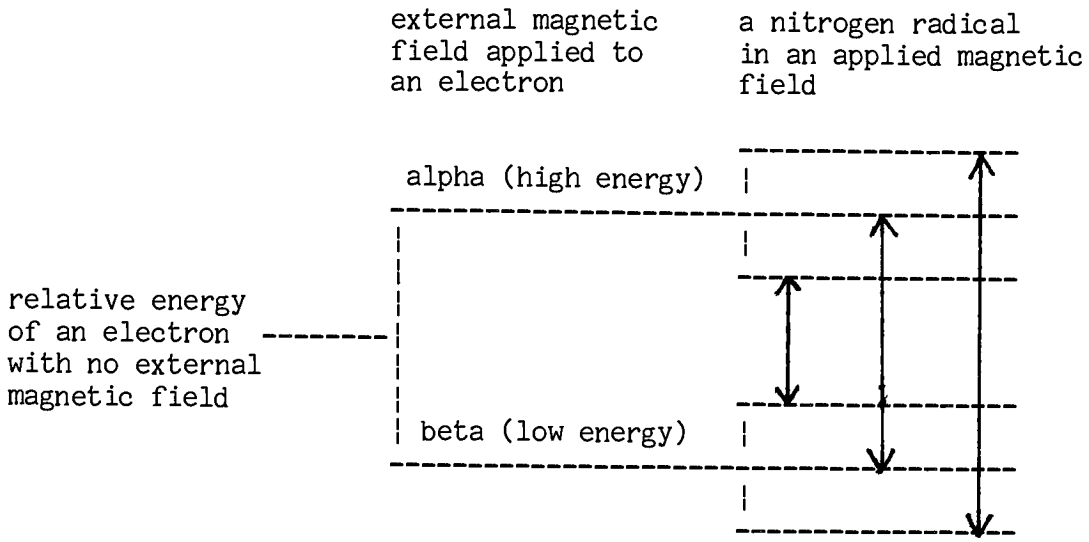


The structure of these copolymers will be determined by IR spectroscopy. The sensitivity of the nitroxide copolymers to molecular oxygen will be analyzed by ESR spectroscopy. Sensitivity will be determined by comparing the ESR spectra of the nitroxide copolymers in an oxygen-rich environment relative to a nitrogen-rich environment. The principle of ESR involves exciting a particle from a low energy state to a high energy state and observing the relative differences in energy[7]. Electrons have a spin quantum number of a half, so they can be aligned with or against an external magnetic field. A high energy state (alpha) is defined when the magnetic field of the particle is oriented against the external magnetic field. A low energy state (beta) is defined when the magnetic field of the particle is oriented with the external magnetic field. Absorption of radiation takes place when the energy of the exciting radiation and the difference in energies of the two states are

equal. The difference in these values is proportional to the magnitude of the external magnetic field. The magnetic field experienced by an unpaired electron becomes more complex if the molecule contains other spin-bearing nuclei, such as nitroxides. A nitroxide is a stable nitrogen centered free radical with the general structure shown below.



Nitroxides are characterized by a strong interaction between the unpaired electron and the spin of the nitrogen nucleus. Nitrogen has a spin quantum number of one, so its nucleus can be oriented in three different alignments relative to the external magnetic field. These three different alignments correspond to three different relative energy levels. Hyperfine interactions are observed when an electron's alpha and beta energy levels are each split into three by the nitrogen nucleus. Thus, excitation of a nitroxide radical can occur with three possible energy transitions, which are illustrated below.



(Vertical arrows show the three possible energy transitions.)

The resulting hyperfine interaction splits the ESR spectra into three absorption peaks, since nitrogen has a spin of one and carbon and oxygen both have zero spin. In practice, the resulting ESR spectra is displayed as the first derivative of the absorption spectra. The shape of these peaks are very sharp and equal in size when the orientations of the spin probes are averaged in respect to the external magnetic field. However, these peaks become broad and uneven as the orientations of the spin probes have less translational and rotational freedom. Spin probes lose mobility in cold temperatures, in viscous solvents, and when bound to large and heavy molecules. Another type of peak distortion is when the three peaks seem to pull together to form one observed signal. This is observed when the concentration of paramagnetic compounds is increased, a consequence of the Heisenberg spin-exchange principle. Figures 2a and 3a show a typical three line spectrum of di-t-butyl nitroxide in low

concentration (millimolar) at room temperature where the nitroxide has freedom of movement and has few interactions with other paramagnetic compounds. Figures 2b and 2c show the broadening effect of lowering the temperature of the nitroxide solution. Figures 3b, 3c, and 3d show the broadening effect of increasing the nitroxide concentration. This broadening effect is the result of collisions between paramagnetic compounds exchanging their spin states. Other paramagnetic compounds which may spin-exchange are molecular oxygen and certain transition metals, which can be found in enzymes.

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The goal of this project is to be able to synthesize a styrene/4-maleimido-TEMPO copolymer that would be sensitive to the presence of dissolved oxygen, but would retain significant free radical activity when introduced to dissolved oxygen. Changes in free radical activity were observed in the copolymers while stored under nitrogen and when introduced to dissolved oxygen. The presence of dissolved oxygen in a nitroxide copolymer solution was qualitatively determined from the amount of peak broadening in the ESR spectra.

EXPERIMENTAL

The IR spectra were run on a Perkin-Elmer FT-IR 1760. IR spectra of all samples were taken in solution cells using chloroform, except in the case of 92/8 [styrene/imide & anhydride] copolymer which used carbon tetrachloride. The ESR spectra were run on a modified spectrometer[8]. 4-Maleimido-TEMPO, 4-amino-TEMPO, and styrene were purchased from Aldrich Chemical Co.. Benzoyl peroxide was obtained from Fisher Scientific. 4-Maleimido-TEMPO was obtained by my own synthetic work and also by the work of Brian Bliss[9]. Both the 92/8 [styrene/maleic anhydride] copolymer and the 50/50 [styrene/maleic anhydride] copolymer were obtained from Monomer - Polymer & Dajac Laboratories, Inc..

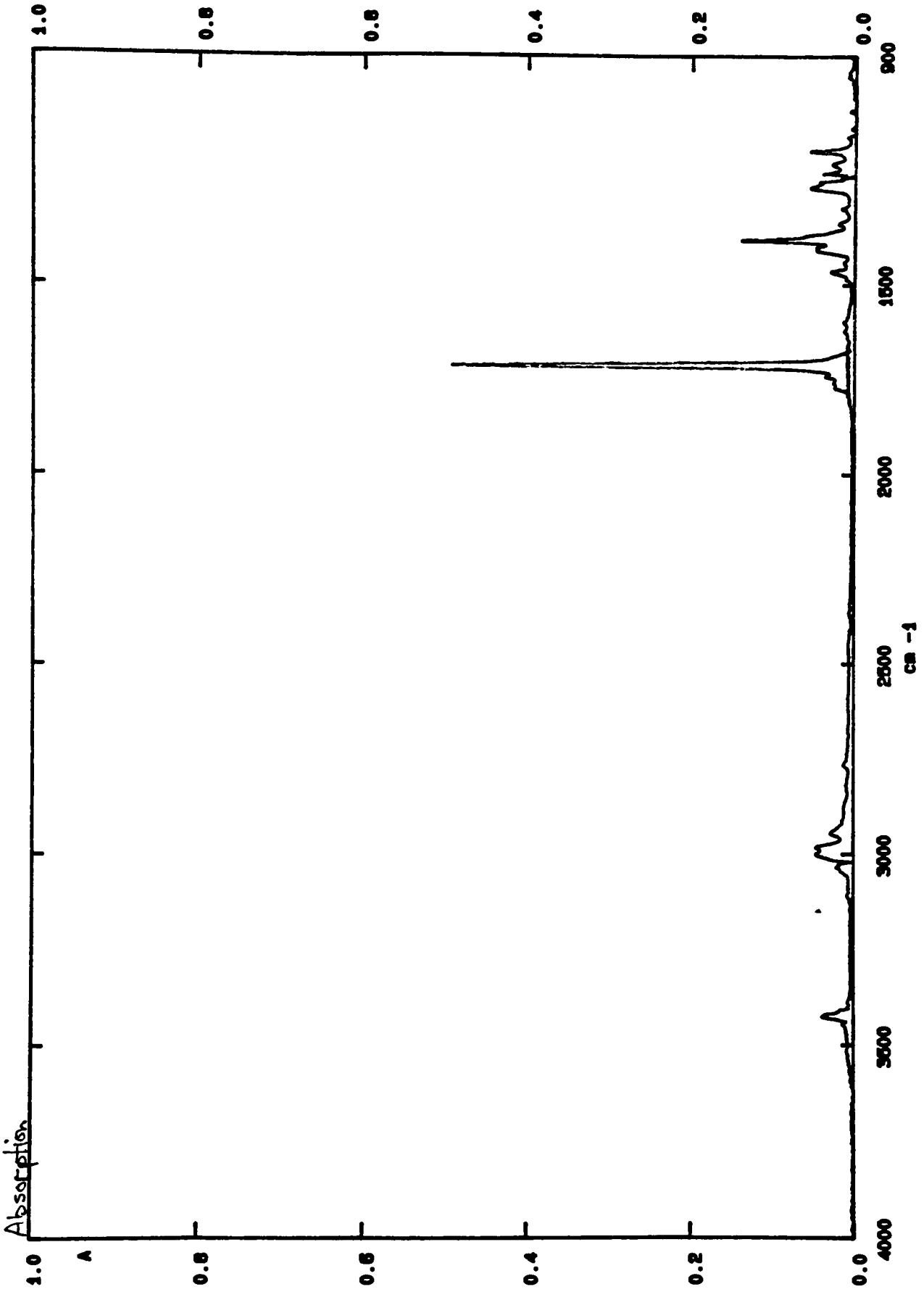
Synthesis of a styrene/4-maleimido-TEMPO copolymer was first attempted by a free radical polymerization of styrene and 4-maleimido-TEMPO monomers. The reaction consisted of heating the two monomers in toluene in the presence of benzoyl peroxide. The 4-maleimido-TEMPO monomer was synthesized in our laboratory by the condensation of maleic anhydride and 4-amino-TEMPO followed by losing the elements of water. Upon the failure of this copolymerization reaction, our strategy changed to begin with a premade styrene/maleic anhydride copolymer and condense it with 4-amino-TEMPO. Various solvents were used to purify the reaction products from the starting materials by selectively dissolving either the product or the starting material.

The Synthesis of 4-Maleimido-TEMPO

2,2,6,6-Tetramethyl-4-aminopiperidine-1-oxyl (0.58 g, 3.4 mmol) was dissolved in a 15 ml anhydrous ether; this solution was added dropwise to a solution of maleic anhydride (0.33 g, 3.4 mmol) in 15 ml anhydrous ether from a dropping funnel. The entire apparatus was wrapped in aluminum foil to keep out light. The mixture was then stirred at room temperature for an hour, and the crude product (0.73 g, 80%) was collected by filtration and washed with several portions of ether. No further attempts to purify the crude material were taken.

To a solution of N-nitroxymaleamic acid (0.96 g, 3.6 mmol) in 15 ml of acetic anhydride, was added anhydrous sodium acetate (0.15 g, 1.8 mmol). The stirred solution was heated to 95-100°C for five hours under nitrogen. The entire apparatus was wrapped in aluminum foil to keep light out. The glass joints were sealed with teflon tape. The acetic anhydride was removed by vacuum distillation. The residue was dissolved in benzene and the remaining solids were removed by filtration. The filtrate was concentrated producing solid material m.p. 98°C, yield 0.67g (69%). A 37 mg (0.15 mmol) sample was dissolved in 30 ml of chloroform in a culture tube which gave the characteristic three line ESR spectra (figure 11 shown in the results and discussion section). The IR spectra (figure 1) is shown below.

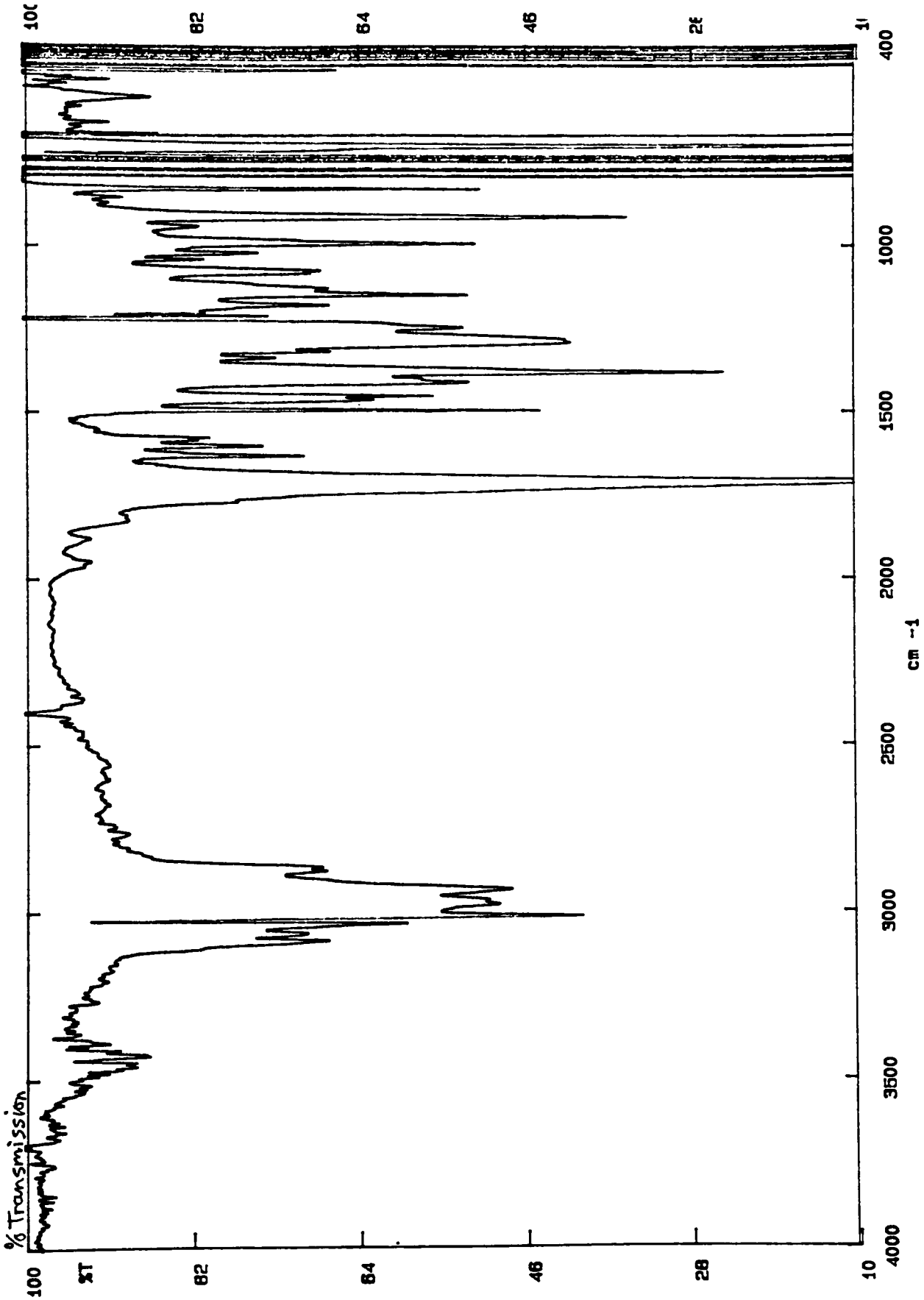
Figure 1: IR of 4-Maleimido-TEMPO



The Copolymerization of Styrene and 4-Maleimido-TEMPO

A batch of 4-maleimido-TEMPO obtained from Brian Bliss[9] (0.54 g, 2.16 mmol) was dissolved in 50 ml of toluene in a three neck round bottom flask equipped with a thermometer and a condenser. The apparatus was carefully cleaned, flame dried, and wrapped in aluminum foil. All glass joints and stoppers were sealed with teflon tape. Nitrogen gas was bubbled through for thirty minutes before heating and continuously while heating. Styrene (5 ml, 43.6 mmol) was added to the solution, and then the solution was heated to 90°C. Benzoyl peroxide (0.030 g, 0.12 mmol) was added to the heated solution and the reaction was kept at 90°C for six hours. The reaction was stopped by placing the reaction flask in an ice bath. The product was dried by vacuum distillation. The IR spectra of the crude material (figure 2 shown below) shows a distinct pattern found in the carbon-hydrogen stretching region of polystyrene. The desired copolymerization was not observed.

Figure 2: IR of the Copolymerization of Styrene and 4-Maleimido-TEMPO



The Synthesis and Purification of 92/8 [Styrene/4-Maleimido-TEMPO] Copolymer

A new strategy was developed for the synthesis of the copolymer by the grafting of 4-amino-TEMPO to a premade 92/8 [styrene/maleic anhydride] copolymer. Included in this synthetic strategy was a means of simultaneously purifying the product in the same reaction pot. This was done by utilizing a particular solvent that would dissolve either the starting materials or the product, but not both. The solubility test procedure used to find such a solvent is described below.

Solubility Tests of the 92/8 Copolymers and 4-Amino-TEMPO:

Five milligram samples of 4-amino-TEMPO were placed in 20 ml scintillation vials. Fifty milligram samples 92/8 [styrene/maleic anhydride], 92/8 [styrene/N-nitroxymaleamic acid & diacetic acid], and 92/8 [styrene/4-maleimido-TEMPO & maleic anhydride] copolymers were placed in separate 20 ml scintillation vials. Each sample was mixed with 15 ml of one of the following solvents: n-heptane, petroleum ether, carbon tetrachloride, benzene, toluene, xylenes, chloroform, methylene chloride, diethyl ether, tetrahydrofuran, ethyl acetate, methyl ethyl ketone, acetonitrile, acetone, 1-octanol, 2-methyl-1-propanol, 95% ethanol, methanol, distilled water, acetic acid, and acetic anhydride.

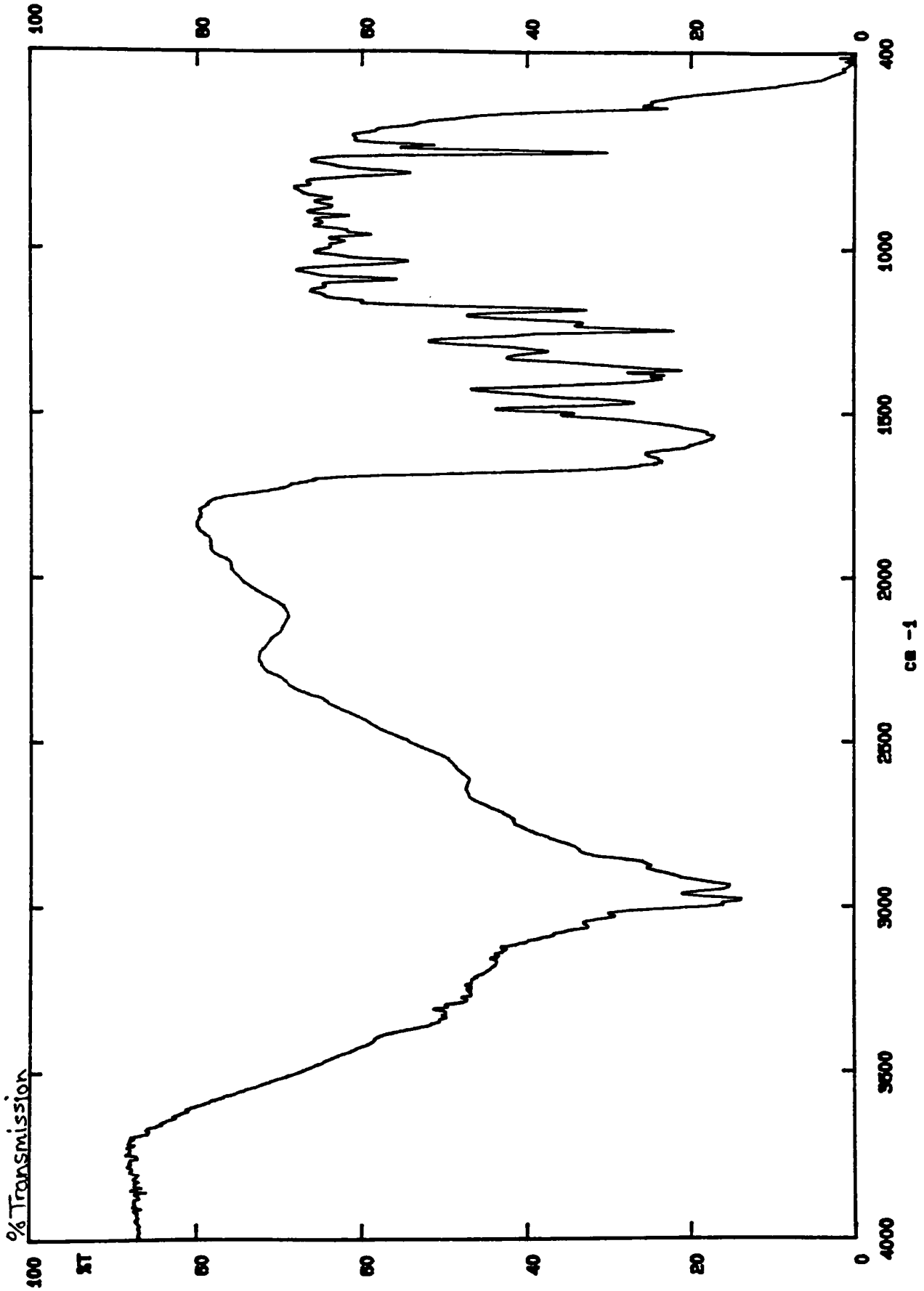
The results showed that carbon tetrachloride dissolves 92/8

[styrene/maleic anhydride] copolymer and 4-amino-TEMPO, but it does not dissolve 92/8 [styrene/N-nitroxymaleamic acid]. Futhermore, carbon tetrachloride dissolves 92/8 [styrene/4-maleimido-TEMPO].

Reaction:

92/8 [Styrene/ maleic anhydride] copolymer (1.940 g, 1.50 mmol of maleic anhydride) was dissolved in 50 ml carbon tetrachloride. 2,2,6,6-Tetramethyl-4-aminopiperidine-1-oxyl (0.264 g, 1.54 mmol) was dissolved in a 20 ml carbon tetrachloride; this solution was added dropwise to the stirred copolymer solution from a dropping funnel. The glassware was flame dried and the apparatus was wrapped in aluminum foil. The mixture was stirred at room temperature for three hours, then the flask was placed into an ice bath. As the copolymer product separated from the solution, the solvent was decanted. The amide/acid copolymer was then placed in an additional portion of hot carbon tetrachloride to redissolve it. The flask was then placed back into the ice bath and the solvent was removed by filtration and then vacuum distillation yielding 1.567g of product. A 0.245 g (0.17 theoretical mmol of N-nitroxymaleamic acid) sample was dissolved in 30 ml of ethyl acetate in a culture tube (figure 13 shown in the results and discussion section) which gave a slightly broader three line ESR spectra than that of a 5 mg (0.029 mmol) sample of 4-amino-TEMPO dissolved in 30 ml of methanol (figure 11 is also shown in the results and discussion section). The IR spectra (figure 3) is shown below.

Figure 3: IR of 92/8 [Styrene/N-Nitroxymaleamic Acid] Copolymer Using Carbon Tetrachloride for Purification



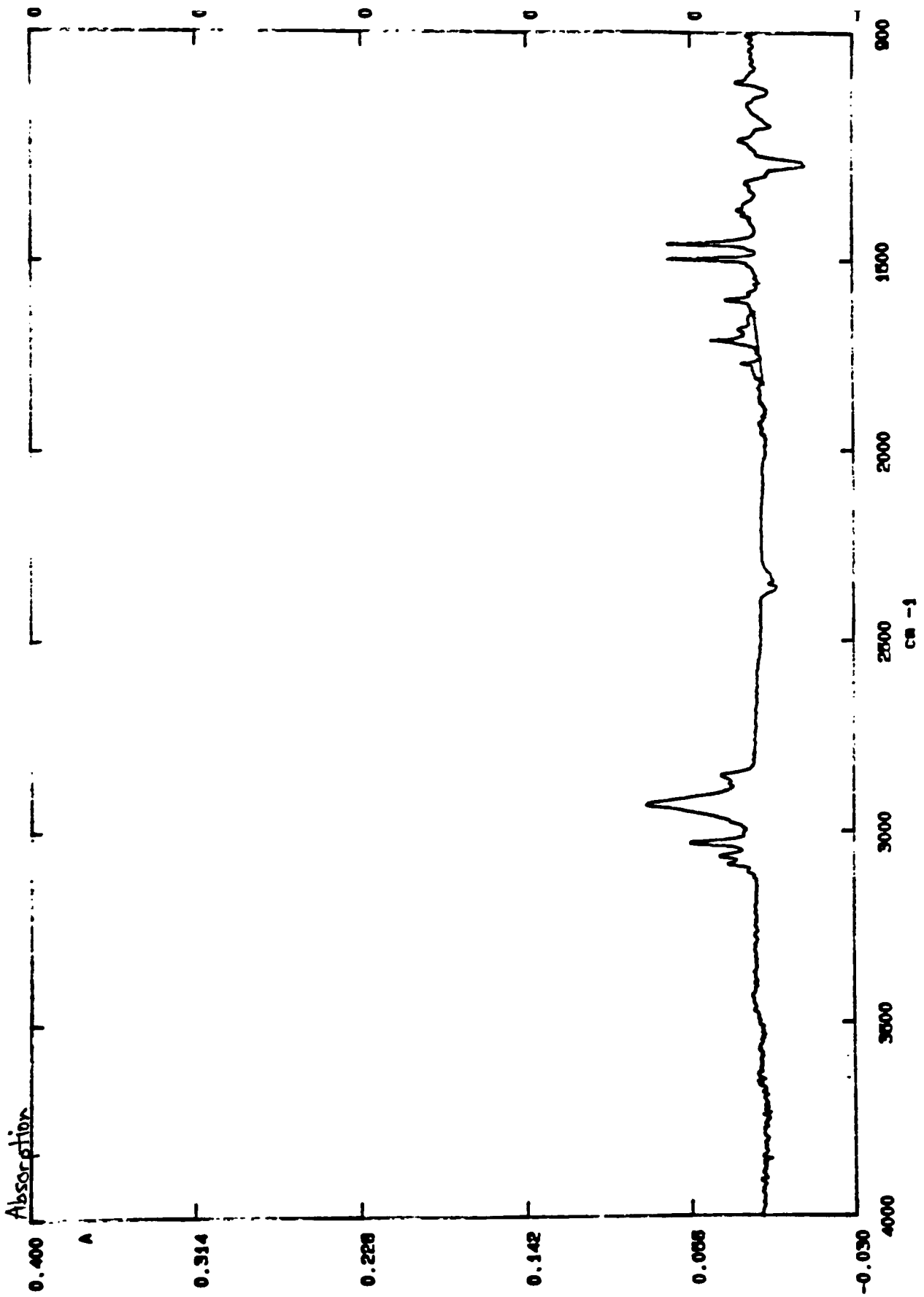
To 1.322g of the above N-nitroxymaleamic acid copolymer was added 50 ml of carbon tetrachloride, and acetic anhydride (0.4 ml, 4.2 mmol) . This was heated to reflux for three hours under nitrogen. The glassware was flame dried and the apparatus was wrapped in aluminum foil. Unreacted amide/acid copolymer was removed by placing the flask into an ice bath and collecting the precipitate by filtration. The solvent was removed from the product by vacuum distillation yielding 0.813g. Analysis of the IR spectra (see Appendix 2) shows that 67% of the original maleic anhydride residues were converted to 4-maleimido-TEMPO residues. A 0.287g (0.20 theoretical mmol of 4-maleimido-TEMPO) sample was dissolved in 30 ml of carbon tetrachloride in a culture tube (figure 15) which gave a slightly broader three line ESR spectra than that of a 5 mg (0.029 mmol) sample of 4-amino-TEMPO dissolved in 30 ml of methanol, and also broader than that of the previous amide/acid spectra (these are shown in the results and discussion section). The IR spectra (figure 4) is shown below.

Additional Solubility Tests:

According to the procedure of Andreis and Veksli[11] which involved similar copolymers and spin probes, methyl ethyl ketone was used as the reaction solvent and cold n-heptane was used to precipitate the reaction products. Another solubility test was performed on all reaction materials and products to see if such a purification method would be useful.

25 Milligrams samples of 4-amino-TEMPO, 92/8 [styrene/maleic anhydride], 92/8 [styrene/N-nitroxymaleamic acid], 92/8 [styrene/4-maleimido-TEMPO], and 50/50 [styrene/maleic anhydride], 50/50 [styrene/N-nitroxymaleamic acid], 50/50 [styrene/4-maleimido-TEMPO] were placed into individual scintillation vials and mixed with 2 ml of methyl ethyl ketone. Once the samples had completely dissolved, 8 ml portions of cold n-heptane were added to each sample. For each sample, there was immediate precipitation of white-orange crystals. Conclusively, n-heptane did not selectively precipitate only the product in each step of the reaction.

Figure 4: IR of 92/8 [Styrene/4-Maleimido-TEMPO & Maleic Anhydride] Copolymer Using Carbon Tetrachloride for Purification



The Synthesis and Purification of 50/50 [Styrene/4-Maleimido-TEMPO] Copolymer

The synthetic strategy described in the procedure for 92/8 [styrene/4-maleimido-TEMPO] copolymer was used again to make a 50/50 [styrene/4-maleimido-TEMPO] copolymer and to simultaneously purify it in the same reaction pot by the use of a particular solvent. The solubility test procedure is described below.

Solubility Tests of the 50/50 Copolymers and 4-Amino-TEMPO:

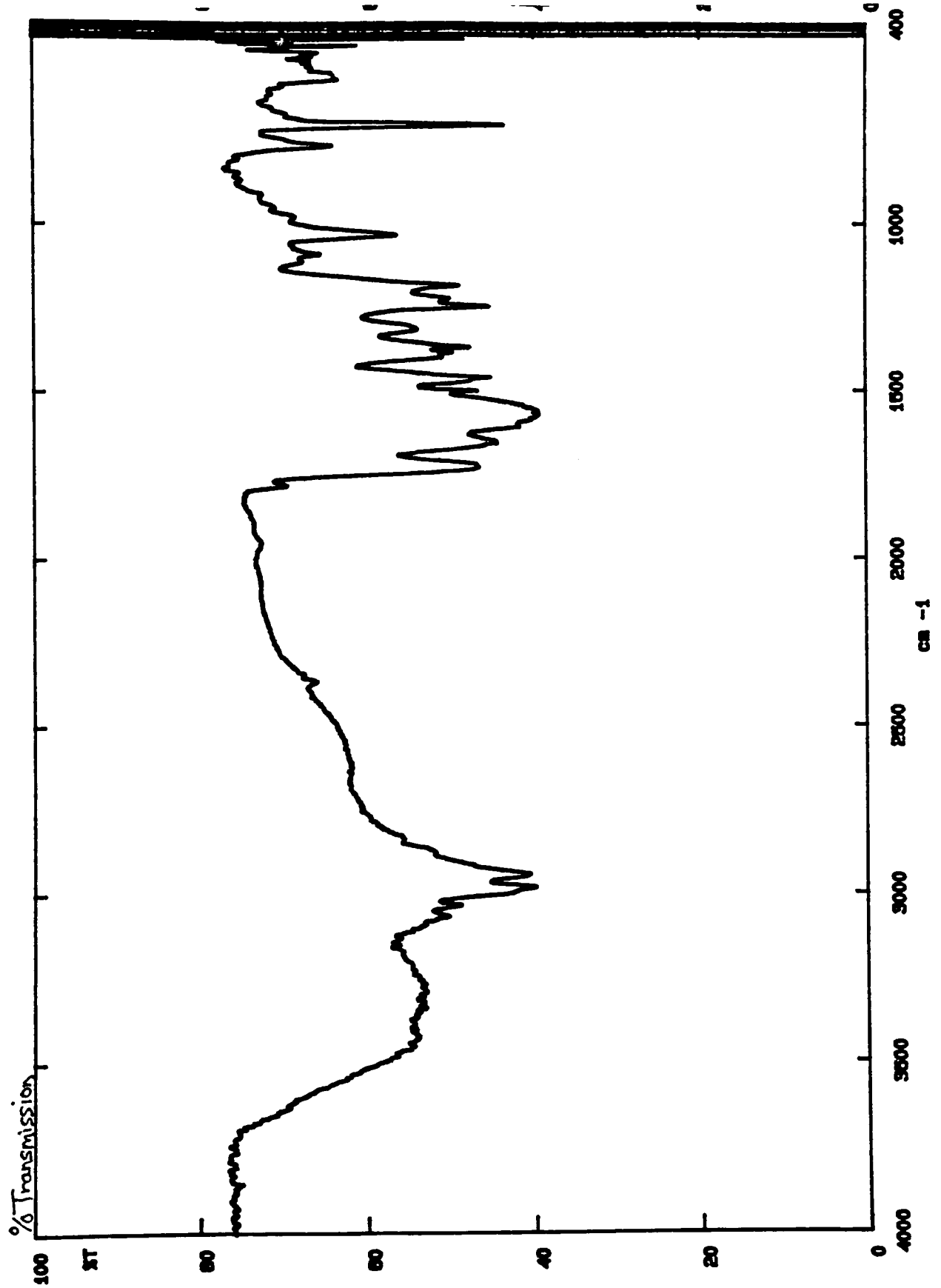
Fifty milligram samples 50/50 [styrene/maleic anhydride], 50/50 [styrene/N-nitroxymaleamic acid], and 50/50 [styrene/4-maleimido-TEMPO] copolymers were placed in separate 20 ml scintillation vials. Each sample was mixed with 15 ml of one of the following solvents: n-heptane, petroleum ether, carbon tetrachloride, benzene, toluene, xylenes, chloroform, methylene chloride, diethyl ether, tetrahydrofuran, acetophenone, ethyl acetate, methyl ethyl ketone, acetonitrile, acetone, methanol, acetic acid, and acetic anhydride.

Of the solvents tested, acetonitrile, tetrahydrofuran, and ethyl acetate would dissolve 4-amino-TEMPO and 50/50 [styrene/maleic anhydride], but not 50/50 [styrene/N-nitroxymaleamic acid]. Furthermore, these solvents dissolve 50/50 [styrene/4-maleimido-TEMPO].

Reaction 1:

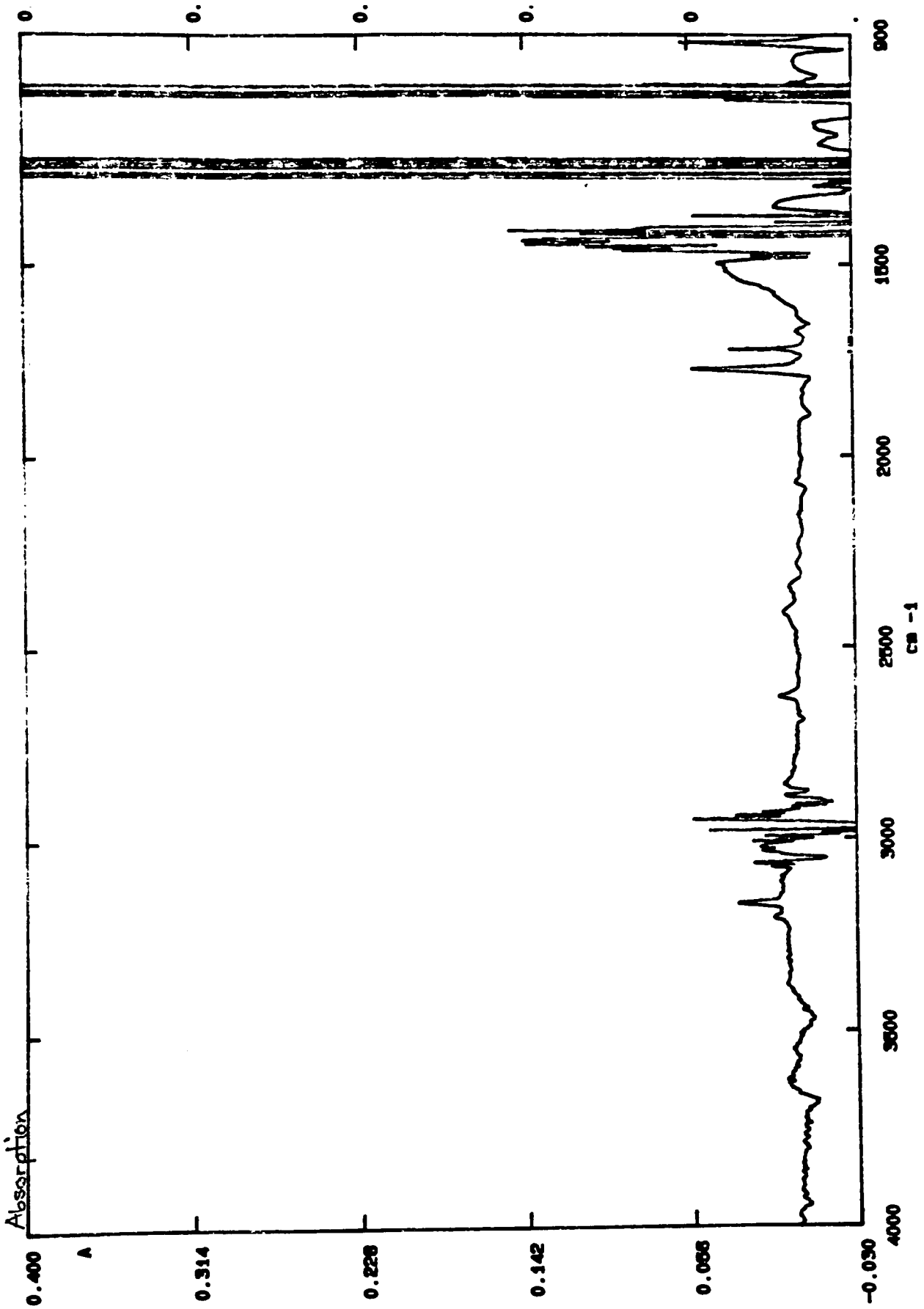
50/50 [Styrene/ maleic anhydride] copolymer (0.303 g, 1.5 mmol of maleic anhydride) was dissolved in 50 ml of tetrahydrofuran. The flask was flame dried and the apparatus was wrapped in aluminum foil. 2,2,6,6-Tetramethyl-4-aminopiperidine-1-oxyl (0.264 g, 1.54 mmol) was dissolved in a 20 ml tetrahydrofuran; this solution was added dropwise to the stirred copolymer solution from a dropping funnel. The mixture was then stirred at room temperature for three hours, and the product was purified by filtration and washed with additional tetrahydrofuran. The solvent was then removed by vacuum distillation yielding 0.212g. The IR spectra (figure 5) is shown below.

Figure 5: IR of 50/50 [Styrene/N-Nitroxymaleamic Acid] Copolymer Using Tetrahydrofuran for Purification



To 0.212g of the above N-nitroxymaleamic acid copolymer was added 50 ml tetrahydrofuran, and acetic anhydride (0.4 ml, 4.2 mmol). This was heated to reflux for three hours under nitrogen. The glassware was flame dried and the apparatus was wrapped in aluminum foil. Unreacted amide/acid copolymer was removed by filtration. The acetic anhydride and tetrahydrofuran were removed by vacuum distillation yielding 0.094g of product. The IR spectra (figure 6) is shown below.

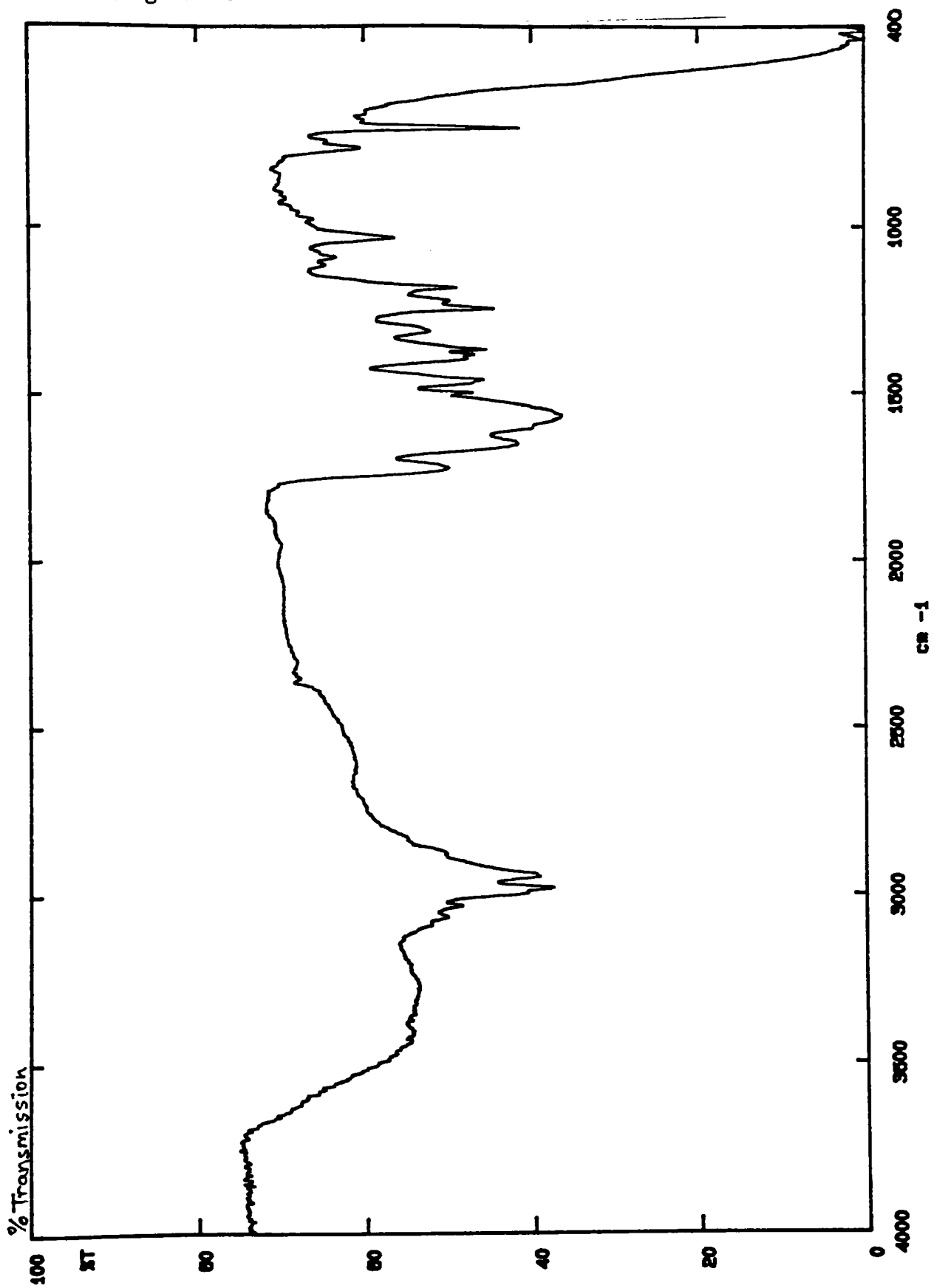
Figure 6: IR of 50/50 [Styrene/4-Maleimido-TEMPO & Maleic Anhydride] Copolymer Using Tetrahydrofuran for Purification



Reaction 2:

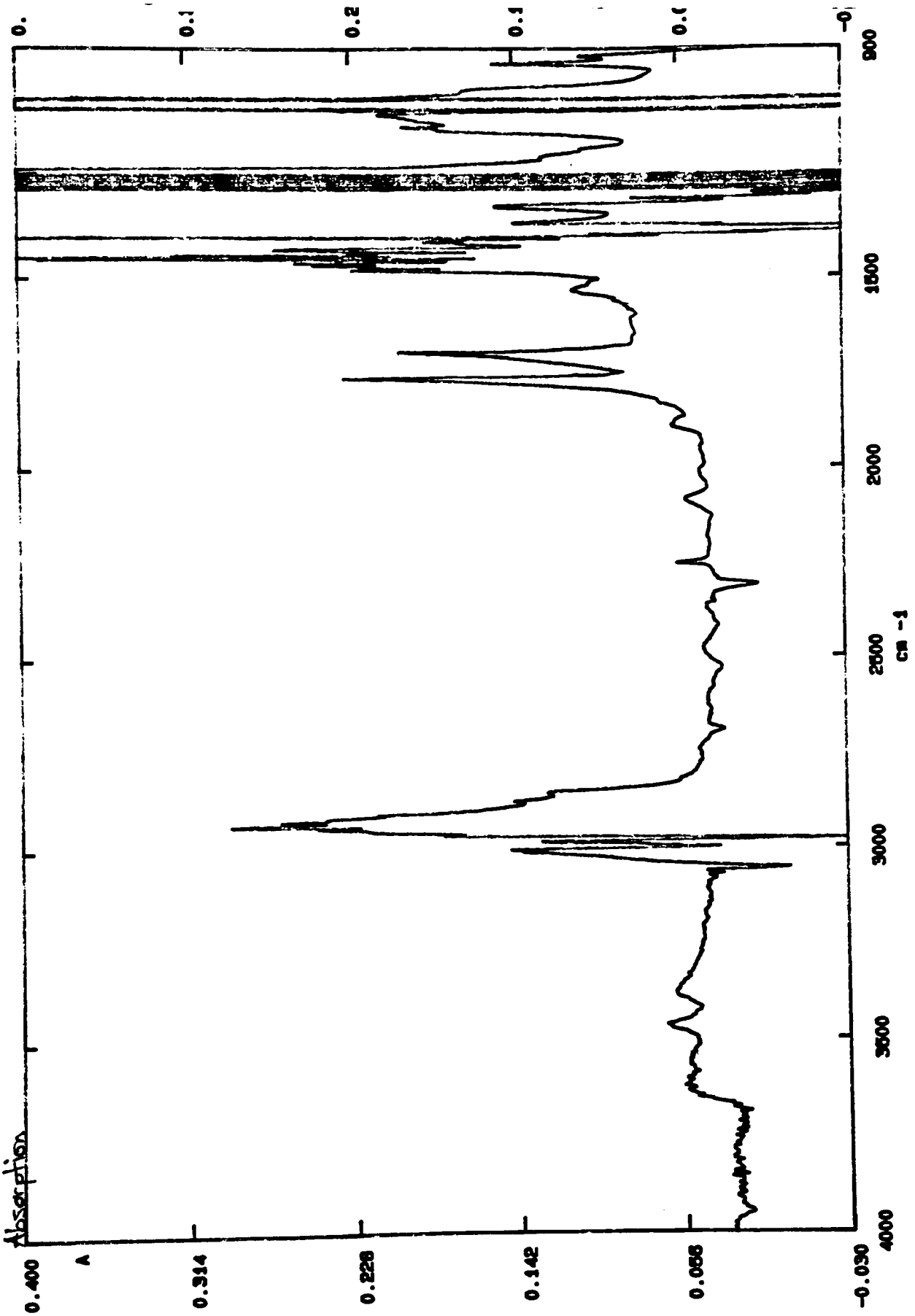
50/50 [Styrene/ maleic anhydride] copolymer (0.305 g, 1.5 mmol of maleic anhydride) was dissolved in 50 ml acetonitrile. 2,2,6,6-Tetramethyl-4-aminopiperidine-1-oxyl (0.262 g, 1.5 mmol) was dissolved in a 20 ml acetonitrile; this solution was added dropwise to the stirred copolymer solution from a dropping funnel. The mixture was then stirred at room temperature for three hours, and the product was purified by filtration and washed with additional acetonitrile. The solvent was removed by vacuum distillation yielding 0.177g of product. The IR spectra (figure 7) is shown below.

Figure 7: IR of 50/50 [Styrene/N-Nitroxymaleamic Acid] Copolymer Using Acetonitrile for Purification



To 0.177g of the above N-nitroxymaleamic acid copolymer was added 50 ml of acetonitrile, and acetic anhydride (0.4 ml, 4.2 mmol). This was heated to reflux for three hours under nitrogen. The glassware was flame dried and the apparatus was wrapped in aluminum foil. Unreacted amide/acid copolymer was removed by filtration. The acetic anhydride and acetonitrile were removed by vacuum distillation yielding 0.118g of product. The IR spectra (figure 8) is shown below.

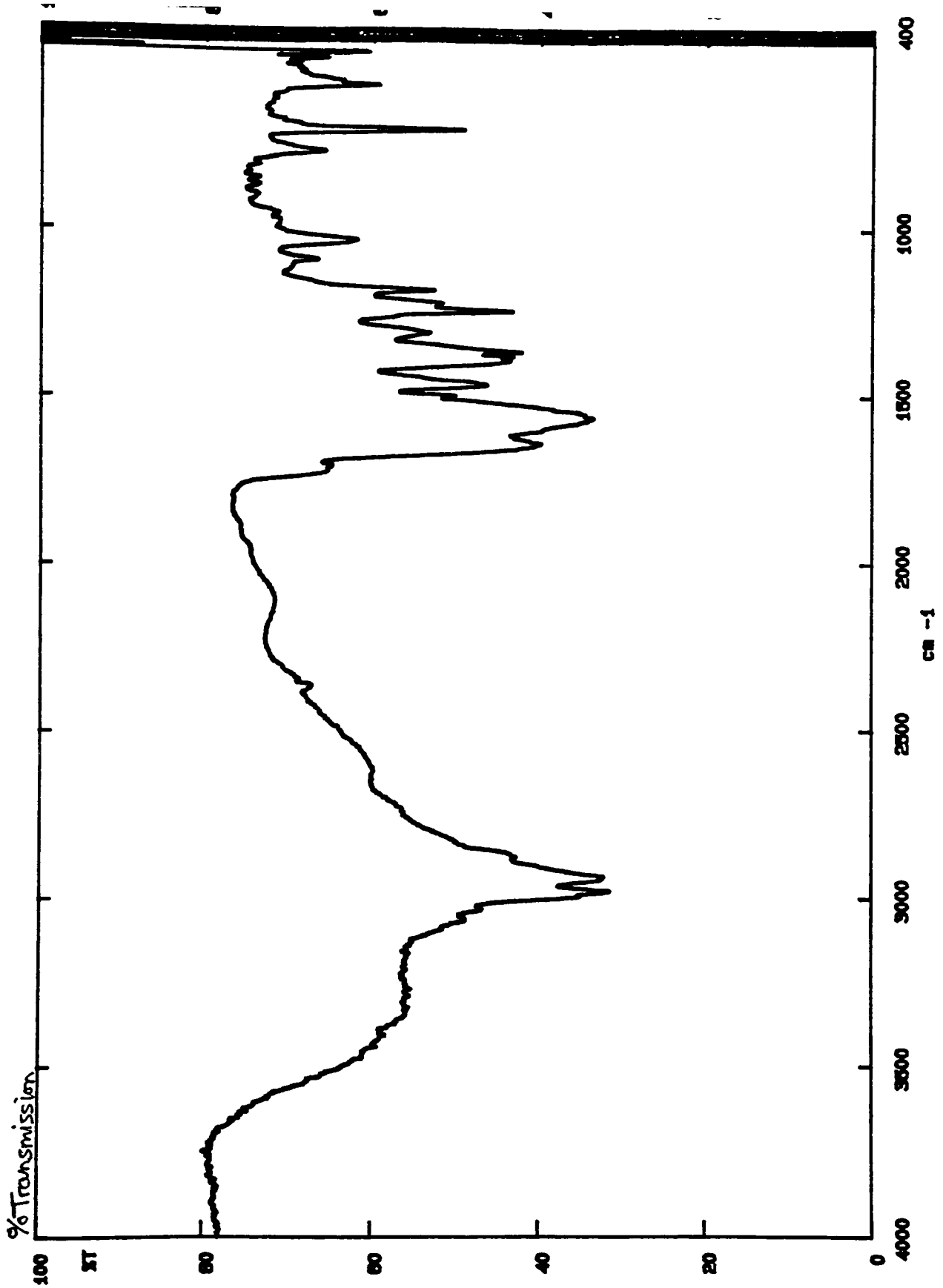
Figure 8: IR of 50/50 [Styrene/4-Maleimido-TEMPO & Maleic Anhydride] Copolymer Using Acetonitrile for Purification



Reaction 3:

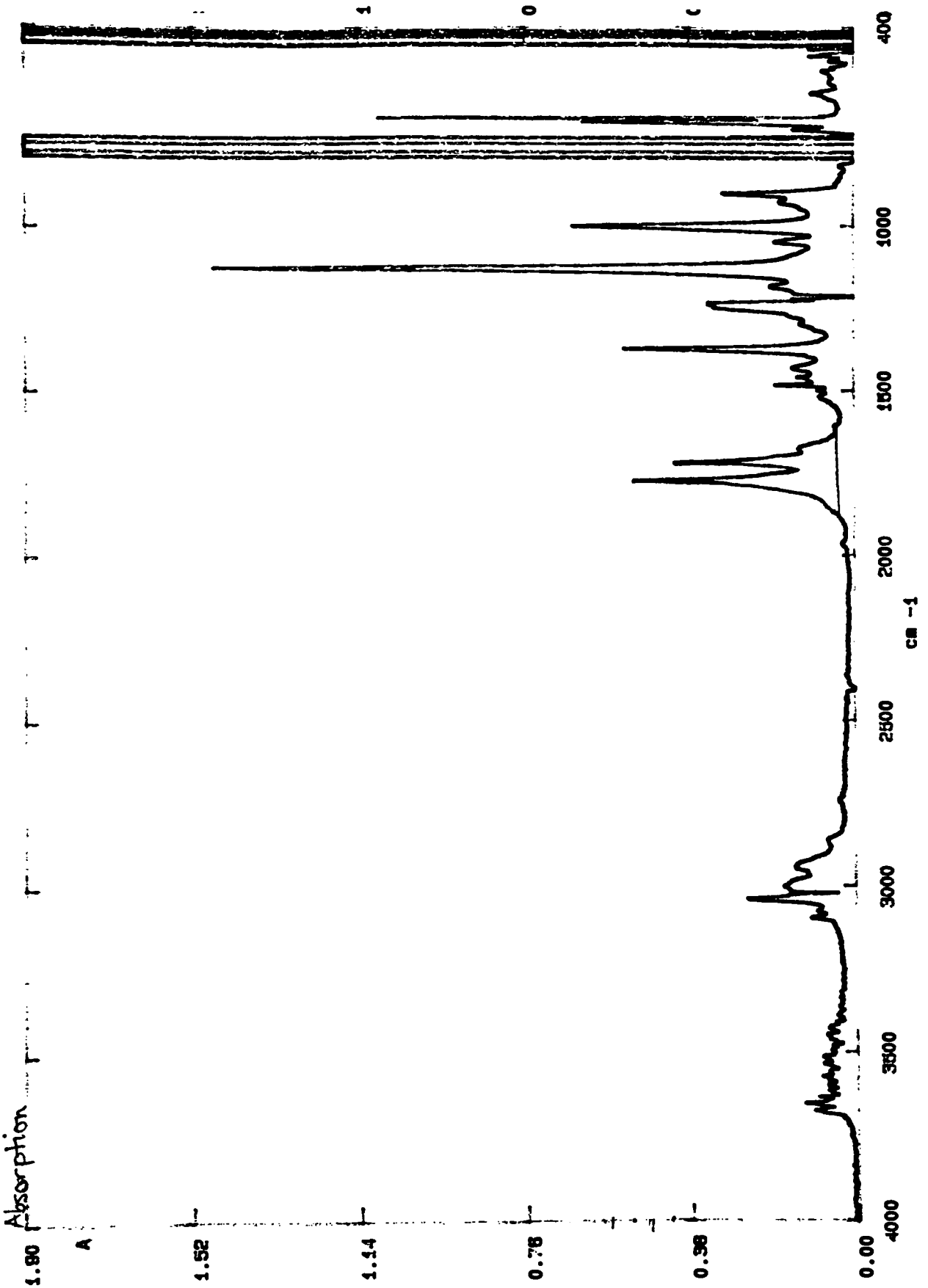
50/50 [Styrene/ maleic anhydride] copolymer (0.302 g, 1.5 mmol of maleic anhydride) was dissolved in 50 ml ethyl acetate. 2,2,6,6-Tetramethyl-4-aminopiperidine-1-oxyl (0.263 g, 1.5 mmol) was dissolved in a 20 ml ethyl acetate; this solution was added dropwise to the stirred copolymer solution from a dropping funnel. The glassware was flame dried and the apparatus was wrapped in aluminum foil. The mixture was then stirred at room temperature for three hours, and the product was purified by filtration and washed with additional ethyl acetate. The solvent was removed by vacuum distillation yielding 0.266g of product. A 0.012 g (0.058 theoretical mmol of N-nitroxymaleamic acid) sample was dissolved in 30 ml of methanol in a culture tube (figure 17 in the results and discussion section) which gave a slightly broader three line ESR spectra than that of a 5 mg (0.029 mmol) sample of 4-amino-TEMPO dissolved in 30 ml of methanol (this is shown in the results and discussion section). The IR spectra (figure 9) is shown below.

Figure 9: IR of 50/50 [Styrene/N-Nitroxymaleamic Acid] Copolymer Using Ethyl Acetate for Purification



To 0.254g of the above N-nitroxymaleamic acid copolymer was added 50 ml of ethyl acetate, and acetic anhydride (0.4 ml, 4.2 mmol) . This was heated to reflux for three hours under nitrogen. The glassware was flame dried and the apparatus was wrapped in aluminum foil. Unreacted amide/acid copolymer was removed by filtration. The acetic anhydride and ethyl acetate were removed by vacuum distillation yielding 0.157g of product. IR analysis of this product (see Appendix 2) shows that 40% of the original maleic anhydride residues were converted to 4-maleimido-TEMPO residues. A 0.011 g (0.052 theoretical mmol of 4-maleimido-TEMPO) sample was dissolved in 30 ml of ethyl acetate in a culture tube (figure 19) which gave a slightly broader three line ESR spectra than that of a 5 mg (0.029 mmol) sample of 4-amino-TEMPO dissolved in 30 ml of methanol, and also broader than that of the previous amide/acid spectra (this is shown in the results and discussion section). The IR spectra (figure 10) is shown below.

Figure 10: IR of 50/50 [Styrene/4-Maleimido-TEMPO & Maleic Anhydride] Copolymer Using Ethyl Acetate for Purification



RESULTS AND DISCUSSION

The purpose of this project was to investigate methods of synthesis of spin probe-grafted copolymers. Two reaction pathways were explored in order to reach this goal. In addition, 4-maleimido-TEMPO was synthesized to obtain enough of it for the copolymerization reactions.

The Synthesis of 4-Maleimido-TEMPO

The success of this experiment was due mostly to the precautions taken to preserve the nitroxide. The use of nitrogen gas to flush oxygen out of the reaction vessel was most effective. Before each step of the reaction was begun, the reaction vessel was flushed with nitrogen, and all of the glass joints and stoppers were sealed with teflon tape. The refluxing step included a continuous flow of nitrogen through the apparatus while heating at 95-100°C. Both the amide/acid and imide products were orange, as was the nitroxide starting material. The melting point of 4-maleimido-TEMPO was in good agreement with the melting point given in the procedure of Hamilton and McConnell[6]. ESR spectra of this product showed a distinctive three line spectrum which proved that the nitroxide survived the reaction conditions.

Additional experiments obtained progressively better yields. In the

production of N-nitroxy-maleamic acid, an important factor that increased yields was the time span of the addition of 4-amino-TEMPO to maleic anhydride, which could be done by dissolving it in more solvent in the dropping funnel. Yields of also improved when maleic anhydride was added in excess, and also by increasing the amount of time of stirring the solutions. In the conversion of the nitroxide amide/acid to 4-maleimido-TEMPO in step two of the reaction, yields improved when the solution was heated longer. The overall yield of the 4-maleimido-TEMPO experiment described in the synthesis section was 55%.

The Copolymerization of Styrene and 4-Maleimido-TEMPO

Copolymer [styrene/4-maleimido-TEMPO] can be synthesized by the copolymerization of styrene and 4-maleimido-TEMPO monomers. The procedure for this reaction was suggested by Dr. Andreas Langner in conversation[10]. The reaction consisted of styrene (colorless) and 4-maleimido-TEMPO (red) monomers and benzoyl peroxide as the free radical initiator. The nitroxide free radical in TEMPO is too stable to induce the polymerization that can be achieved from benzoyl peroxide. A sample of 4-maleimido-TEMPO was dissolved in toluene in a round bottomed flask, and then a portion of styrene was added to it. Benzoyl peroxide was added after the two monomers were in solution to reduce the chances of homopolymerization. The mixture was then heated for several hours so

that the benzoyl peroxide alters its structure to expose a free radical. Because of this high reactivity, the reaction apparatus was continually flushed with nitrogen gas to prevent air oxidation. Also, the apparatus was wrapped with aluminum foil because TEMPO is light sensitive. The 4-maleimido-TEMPO used was synthesized by Brian Bliss[9]. Extra measures to clean the apparatus were taken in order to allow the benzoyl peroxide to react more with the monomers than with contaminants. The reaction solution had only a small change in appearance after five hours of refluxing. The deep red crystals obtained showed very distinctive peaks in the infrared region that were similar to those of the starting materials. It was then concluded that this copolymerization method was unsatisfactory.

The Synthesis and Purification of 92/8 [Styrene/4-Maleimido-TEMPO] Copolymer

Using the synthetic procedure of 4-maleimido-TEMPO from 4-amino-TEMPO and maleic anhydride mentioned previously, we created a copolymer of 92/8 [styrene/ 4-maleimido-TEMPO] from 4-amino-TEMPO and a copolymer of 92/8 [styrene/maleic anhydride]. The only differences between these two procedures was a change of solvent and that the maleic anhydride starting material was already incorporated into a styrene copolymer. Polystyrene is relatively inert and may prove to adequately protect the

nitroxide from in vivo degradation.

There was some difficulty in utilizing acetic anhydride as the solvent in the second step of the procedure because it was hard to separate from the copolymer products. The development of a purification process was the most important addition to the overall procedure. Naturally, it is essential to have purification to achieve an accurate analysis of reaction products. If no purification steps are taken, then IR spectra could show peaks of both starting materials and the reaction products. The purification method of Andreis and Veksli[11], which experimented with similar copolymers and spin probes, used methyl ethyl ketone as the reaction solvent and cold n-heptane to precipitate their reaction products. Methyl ethyl ketone did dissolve all of the copolymers researched in this project, but cold n-heptane was not selective and precipitated all of the reaction starting copolymers and the 4-amino-TEMPO as well as the product copolymers. A solvent was needed which would dissolve either the starting material or the reaction product, but not both. An extensive solubility test was performed on the all of the reaction materials and the results are described in Table 1.

Table 1. Solubilty Tests of the 92/8 Copolymers and 4-Amino-TEMPO

solvent (boiling point °C)	4-amino-TEMPO	92/8 [styrene/ maleic anhydride]	92/8 [styrene/ N-nitroxy-maleamic acid & diacetic acid]	92/8 [styrene/ 4-maleimido-TEMPO & maleic anhydride]
n-heptane (98)	insoluble	insoluble	insoluble	insoluble
petroleum ether (30-60)	soluble	insoluble	insoluble	
carbon tetrachloride (77)	soluble	soluble	swells*	soluble
benzene (80)		very soluble	very soluble	
toluene (122)	soluble	soluble	soluble	
xylenes (137-144)	soluble	soluble	soluble	
chloroform (61)	soluble	soluble	very soluble	
methylene chloride (41)	soluble	soluble	very soluble	
diethyl ether (35)	soluble	swells	swells	
tetrahydrofuran (67)	soluble	soluble	insoluble	

Table 1 (continued).

solvent (boiling point °C)	4-amino-TEMPO	92/8 [styrene/ maleic anhydride]	92/8 [styrene/ N-nitroxy-maleamic acid & diacetic acid]	92/8 [styrene/ 4-maleimido-TEMPO & maleic anhydride]
ethyl acetate (77)	soluble	very soluble	very soluble	
methyl ethyl ketone (79)	soluble	very soluble	very soluble	
acetonitrile (81)	soluble	swells	insoluble	
acetone (56)	soluble	suspension	swells	
1-octanol (194-196)		insoluble	insoluble	
2-methyl-1-propanol (108)		insoluble	insoluble	
95% ethanol (78)	soluble	insoluble	insoluble	
methanol (65)	soluble	insoluble	insoluble	
distilled water (100)	soluble	insoluble	insoluble	
acetic acid (118)		insoluble	swells	
acetic anhydride (138-140)			soluble	soluble

* -- This was the only copolymer that dissolved in a hot solvent.

Fortunately, 4-amino-TEMPO dissolved well in most any solvent, except n-heptane. Carbon tetrachloride dissolved both 4-amino-TEMPO and 92/8 [styrene/maleic anhydride] copolymer. However, carbon tetrachloride did not dissolve the 92/8 [styrene/N-nitroxy-maleamic acid & diacetic acid] copolymer. When carbon tetrachloride was used as the solvent for step one of the reaction, the amide/acid copolymer product precipitated as a thick orange gel which had swelled to absorb all of the solvent in the reaction flask. When the flask was placed into an ice bath, the solvent separated from the amide/acid copolymer as it contracted. Evidently, this procedure purified the amide/acid copolymer because all of the starting materials were completely soluble in carbon tetrachloride, which was removed from the precipitated product. All that was needed to separate the amide/acid copolymer from the starting materials was to place the reaction flask into an ice bath and then decant the solvent as the amide/acid copolymer shrunk.

When heated in carbon tetrachloride, the amide/acid copolymer redissolved and was ready for the second step of the reaction. Carbon tetrachloride was used as the reaction solvent in place of acetic anhydride. Only millimolar quantities of acetic anhydride were added to facilitate ring closure to form the imide. No sodium acetate was added. Fortunately, carbon tetrachloride refluxes at 77°C, which was high enough for the formation of the imide and yet low enough to avoid thermal degradation of the nitroxide. Surprisingly, the imide copolymer product of step two was very soluble in carbon tetrachloride at temperatures ranging from 0 to 77°C. This meant that unreacted amide/acid copolymer

could be removed by placing the reaction flask back into an ice bath and filtering it out of the imide solution.

Another solvent, acetonitrile, has the potential to produce the same results. Acetonitrile swells 92/8 [styrene/maleic anhydride] copolymer, so it has the potential to completely dissolve it (perhaps with the addition of another factor which has not yet been discovered). Hot acetonitrile did not dissolve 92/8 [styrene/maleic anhydride], but maybe it would in a sonic bath or by its combination with other solvents; the solubility did not seem to improve with the use of a sonic bath. If the starting copolymer becomes dissolved, the amide/acid copolymer would precipitate as it forms because it is not soluble in acetonitrile. This method would automatically purify the amide/acid copolymer similarly to the way carbon tetrachloride worked.

The analysis of the IR spectra for this new imide copolymer was that the mole fraction of 4-maleimido-TEMPO to maleic anhydride was 67%. The abundance of these two residues were derived by quantitative studies of their unique IR absorbance values. The different IR signals of these residues were identified by a comparison of maleic anhydride and 4-maleimido-TEMPO spectra. Table 2 (shown below) shows the signals of the copolymers which identify the presence of imides and anhydrides. Anhydride groups typically absorb at about 1780 cm^{-1}, and imide groups typically absorb at about 1710 cm^{-1}.

TABLE 2: The carbonyl peaks of the imide and anhydride are very distinctive, so they are the signals which best identify the presence of these compounds.

chemical group type	4-maleimido-TEMPO	maleic anhydride	92/8 styrene/ maleic anhydride	92/8 styrene/ imide & anhydride
anhydride carbonyl	----	1781	1779	1784
imide carbonyl	1711	----	----	1715

Quantification of these groups in the copolymers were made by a comparison of absorbance values to samples of known concentrations of maleic anhydride and 4-maleimido-TEMPO. These calculations are described in detail in Appendix 2.

ESR analysis of both the amide/acid (figure 13) and the imide (figure 15) copolymers, from the procedure that utilized carbon tetrachloride as the solvent system, found that a significant amount of the nitroxide had survived the reaction conditions as well as the introduction of dissolved oxygen. The ESR analysis presented here is for qualitative purposes; no quantitative studies were made to measure nitroxide degradation. Both copolymers were stored under nitrogen and their ESR spectra displayed a three line spectrum that was a little broader than the spectra of free 4-amino-TEMPO (figure 11). This effect was caused by the restricted mobility of the nitroxide as it is bound to the copolymers. The same nitroxide solutions were then bubbled through

with oxygen, which made each ESR spectra noticeably different because of the Heisenberg spin exchange between triplet oxygen and the nitroxide. The ESR spectra of the solution of 4-amino-TEMPO introduced to dissolved oxygen (figure 12) is shown adjacent to the same sample previously stored under nitrogen (figure 11) for purposes of comparison and contrast. The ESR spectra of the amide/acid copolymer solution introduced to dissolved oxygen (figure 14) and that of the imide copolymer (figure 16) are also shown adjacent to their respective spectras of the samples previously stored under nitrogen.

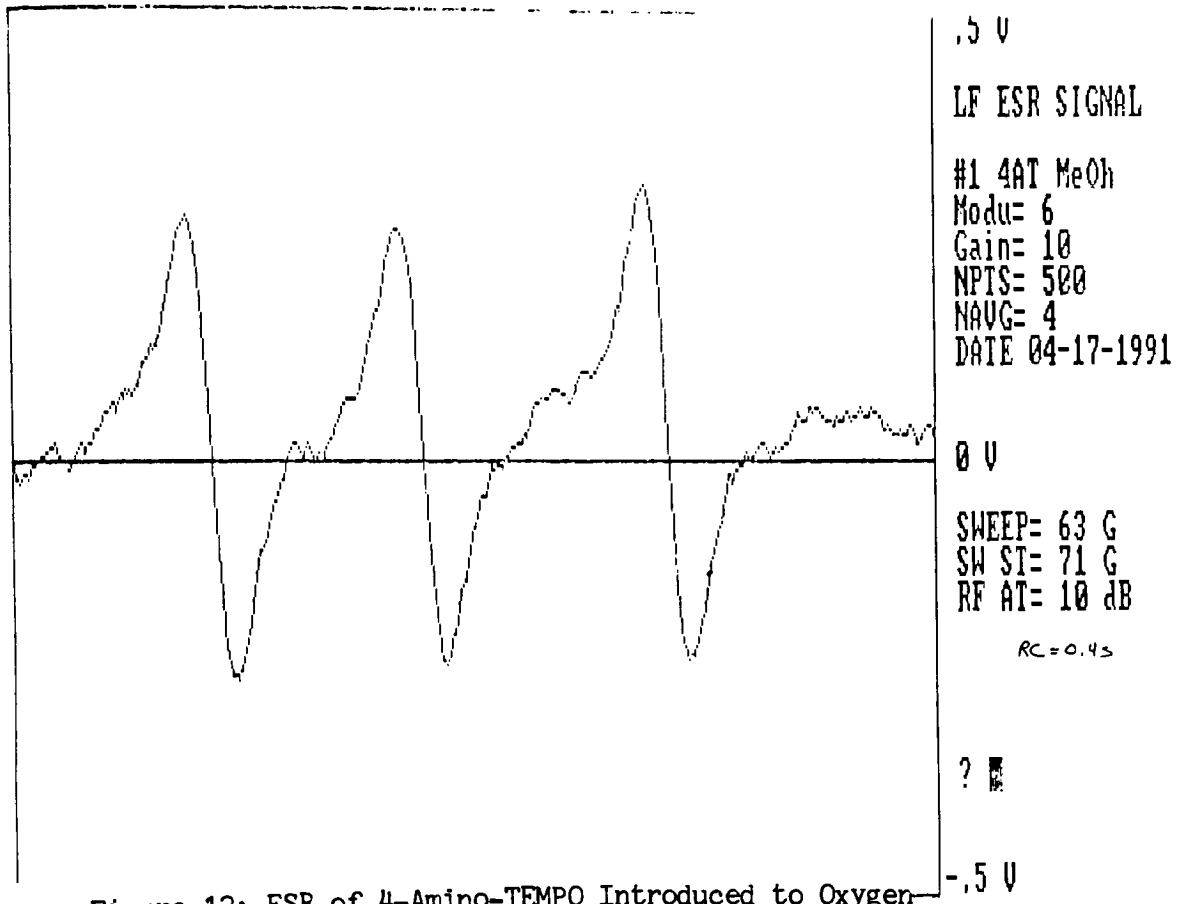


Figure 12: ESR of 4-Amino-TEMPO Introduced to Oxygen

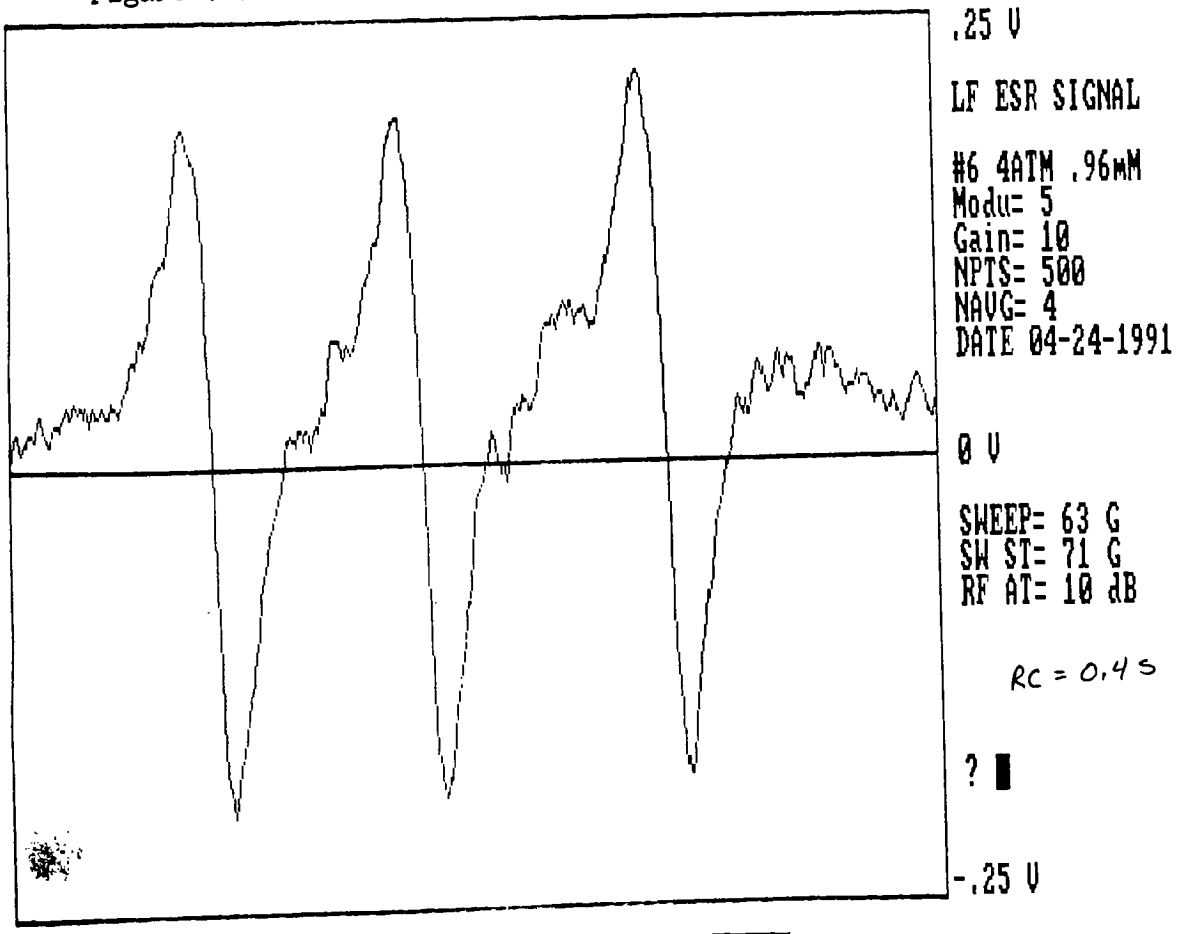


Figure 13: ESR of 92/8 [Styrene/N-Nitroxymaleamic Acid] Copolymer (Using Carbon Tetrachloride for Purification) Stored Under Nitrogen

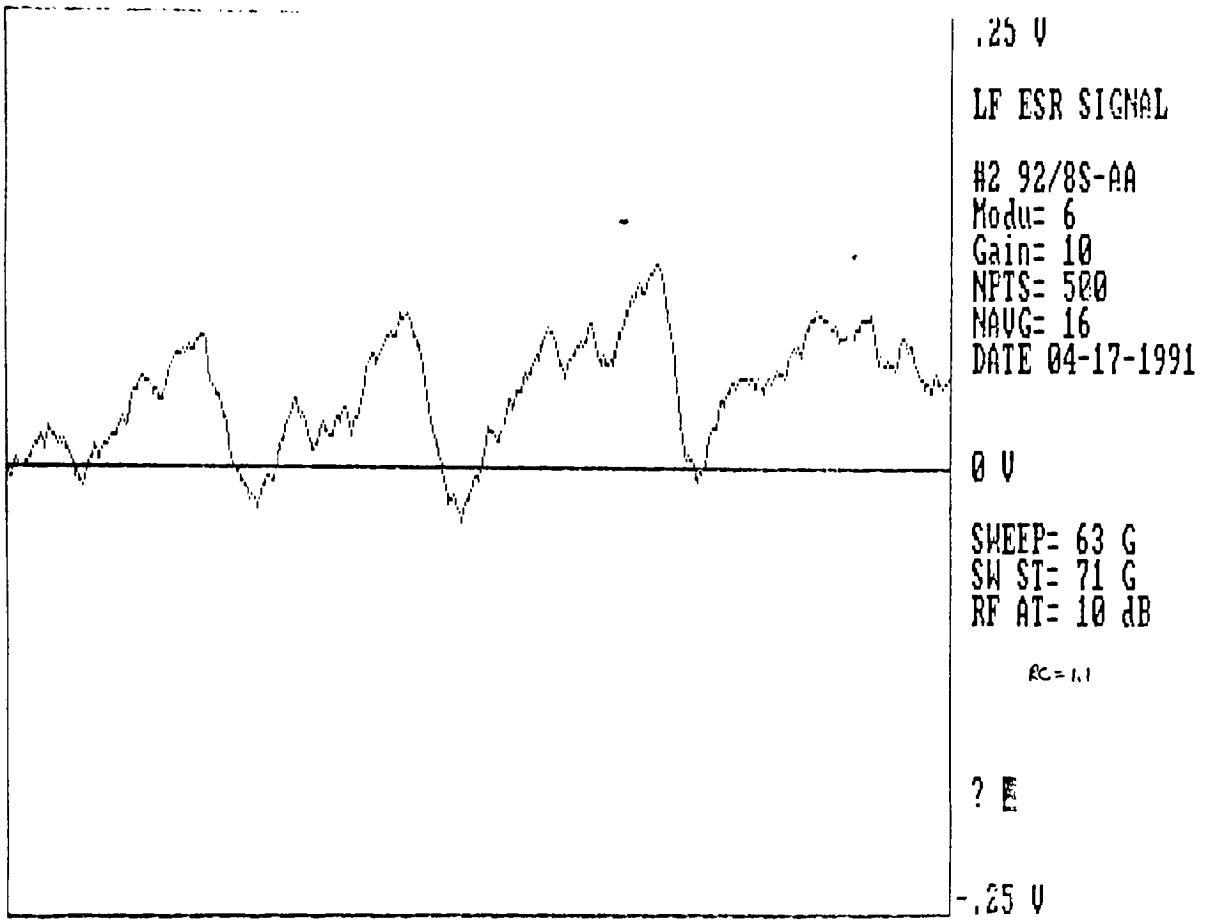


Figure 14: ESR of 92/8 [Styrene/N-Nitroxymaleamic Acid] Copolymer (Using Carbon Tetrachloride for Purification) Introduced to Oxygen

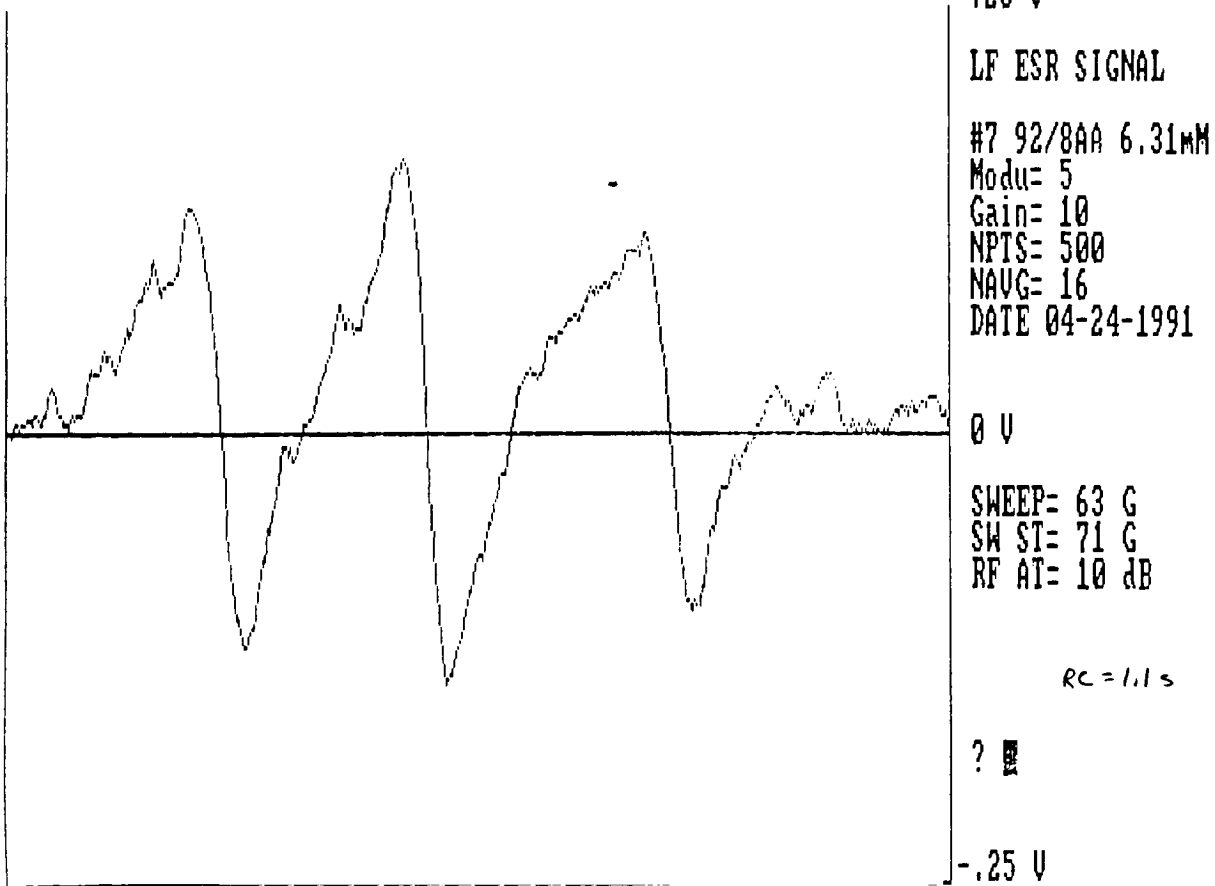


Figure 15: ESR of 92/8 [Styrene/4-Maleimido-TEMPO & Maleic Anhydride] Copolymer (Using Carbon Tetrachloride for Purification) Stored Under Nitrogen

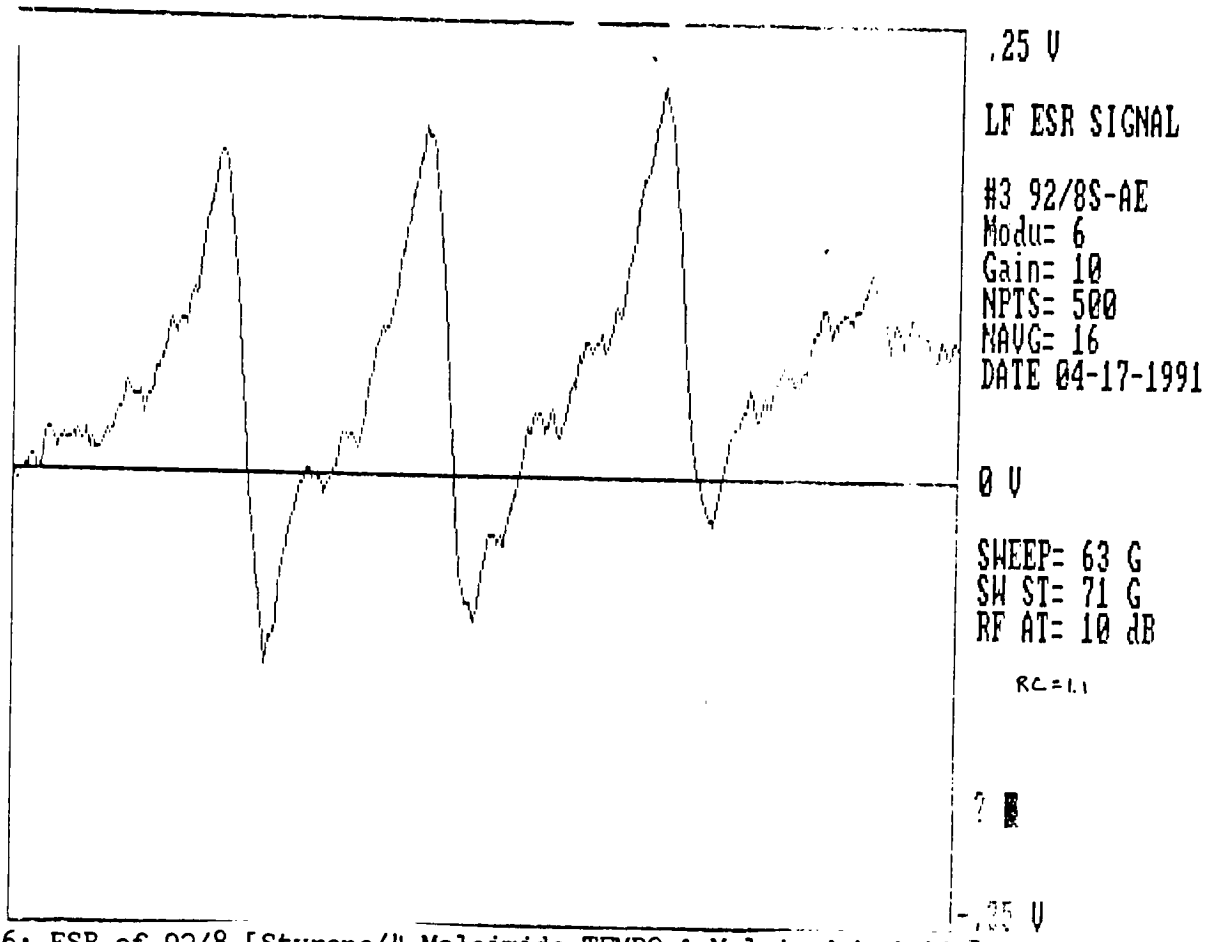
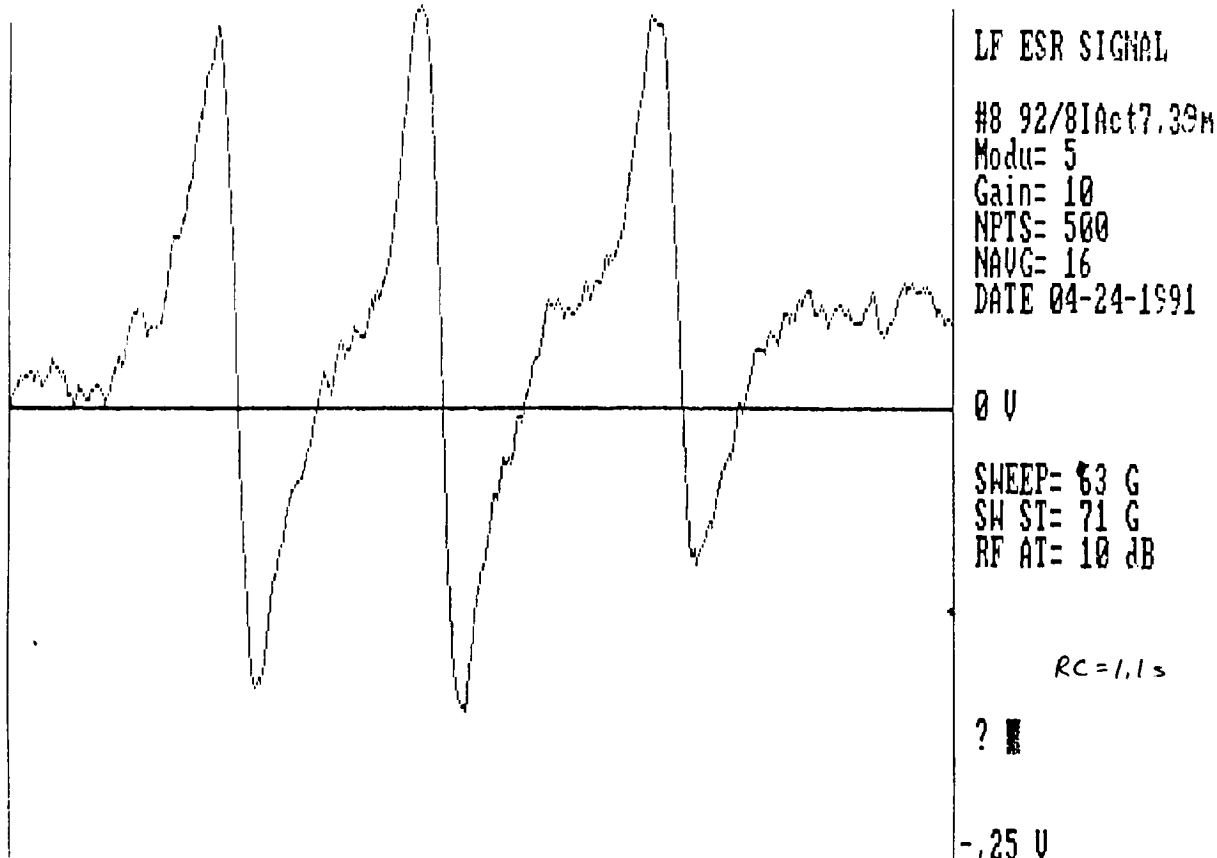


Figure 16: ESR of 92/8 [Styrene/4-Maleimido-TEMPO & Maleic Anhydride] Copolymer (Using Carbon Tetrachloride for Purification) Introduced to Air



The hyperfine splitting and peak to peak widths of these spectra are described in Table 3. The hyperfine splitting is defined by the value in Gauss from the maximum of the first peak to the maximum of the second peak. The peak to peak widths are defined by the value in Gauss from the maximum to the minimum of any particular peak. The values presented in this table are approximate and are intended to demonstrate the signal distortion caused by dissolved oxygen.

Table 3: A comparison of hyperfine splitting (A) and peak to peak widths (Hpp) in Gauss of the three ESR peaks of the 92/8 copolymers and 4-amino-TEMPO when stored under nitrogen, and then introduced to oxygen. The values presented here are approximate due to signal noise and the inherent limitations of using a ruler for making measurements.

		Peak to Peak (ΔHpp) Values and Hyperfine Splitting (A)	
Sample		stored under nitrogen	introduction of oxygen
4-amino-TEMPO	ΔHpp 1	3.43	3.39
	ΔHpp 2	3.50	3.04
	ΔHpp 3	3.04	3.39
	A	14.23	14.48
92/8 [styrene/N-nitroxy-maleamic acid & diacetic acid] copolymer	ΔHpp 1	3.64	3.57
	ΔHpp 2	3.75	2.86
	ΔHpp 3	2.68	3.21
	A	13.59	14.13
92/8 [styrene/4-maleimido-TEMPO & maleic anhydride] copolymer	ΔHpp 1	3.21	2.50
	ΔHpp 2	3.39	3.04
	ΔHpp 3	3.57	3.21
	A	13.59	13.41

The Synthesis and Purification of 50/50 [Styrene/4-Maleimido-TEMPO] Copolymer

With the success of the 92/8 copolymer purification process, further solubility tests were carried out for the 50/50 copolymers. The results of these tests are described in Table 4 (shown below). Amazingly, none of the solvents used the 92/8 copolymer experiments had the same effect on the 50/50 copolymers. However, several other solvents did work well to purify 50/50 copolymer products. Tetrahydrofuran (b.p. 67°C), ethyl acetate (b.p. 77°C), and acetonitrile (b.p. 81°C) worked similarly to purify the reaction product at each step. First, they dissolved the starting materials, precipitated the amide/acid copolymer, and then dissolved the imide copolymer. Just as carbon tetrachloride worked for the 92/8 copolymers, each of these solvents worked to either dissolve the starting copolymer or the product, but not both. Thus, purification was automatically achieved at each step of the reaction. Also, all of these solvents reflux at desired temperatures for imide formation, but low enough to not cause nitroxide degradation. The best of these three solvents was ethyl acetate because it precipitated the amide/acid copolymer as a fine powder rather than a swollen gel as did the other two solvents. This meant that ethyl acetate could be removed from the copolymer more easily.

Table 4. Solubility Tests of the 50/50 Copolymers and 4-Amino-TEMPO

solvent (boiling point °C)	4-amino-TEMPO	50/50 [styrene/ maleic anhydride]	50/50 [styrene/ N-nitroxy-maleamic acid & diacetic acid]	50/50 [styrene/ 4-maleimido-TEMPO & maleic anhydride]
n-heptane (98)	insoluble	insoluble	insoluble	insoluble
petroleum ether (30-60)	soluble	insoluble		
carbon tetrachloride (77)	soluble	insoluble		insoluble
benzene (80)		insoluble	insoluble	
toluene (122)	soluble	insoluble		
xylenes (137-144)	soluble	insoluble		
chloroform (61)	soluble	insoluble	partly soluble	partly soluble
methylene chloride (41)	soluble		partly soluble	insoluble
diethyl ether (35)	soluble	insoluble		
tetrahydrofuran (67)	soluble	very soluble	swells	partly soluble

Table 2 (continued).

solvent (boiling point °C)	4-amino-TEMPO	50/50 [styrene/ maleic anhydride]	50/50 [styrene/ N-nitroxy-maleamic acid & diacetic acid]	50/50 [styrene/ 4-maleimido-TEMPO & maleic anhydride]
acetophenone (202)			soluble in 24 hrs.*	
ethyl acetate (77)	soluble	soluble	insoluble	soluble
methyl ethyl ketone (79)	soluble	very soluble	partly soluble	
acetonitrile (81)	soluble	very soluble	emulsion	partly soluble
acetone (56)	soluble	soluble	partly soluble	
methanol (65)	soluble	soluble in 3 weeks	very soluble	soluble
acetic acid (118)			soluble	partly soluble
acetic anhydride (138-140)			soluble	soluble

* -- This was the only solvent available that dissolved the amide/acid.

Ethyl acetate was the key factor in the successful synthesis of 50/50 imide copolymer. Hot ethyl acetate does not redissolve the amide/acid copolymer, but this did not hinder the formation of the imide which did dissolve in the second step of the reaction. The addition of only millimolar quantities of acetic anhydride (and no addition of sodium acetate) in the second step of the reaction helped minimize the amount of it that remains in the copolymer product. Only trace amounts of unreacted amide/acid copolymer remained undissolved after the reaction was completed, which were removed by filtration.

The analysis of the IR spectra for this new imide copolymer was that the mole fraction of 4-maleimido-TEMPO to maleic anhydride residues was 40%. The abundance of these two residues were derived by quantitative studies of their unique IR absorbance values. The different IR signals of these residues were identified by a comparison of maleic anhydride and 4-maleimido-TEMPO spectra. Table 5 shows the signals of the copolymers which identify the presence of imides and anhydrides. Anhydride groups typically absorb at about 1780 cm^{-1}, and imide groups typically absorb at about 1710 cm^{-1}.

TABLE 5: The carbonyl peaks of the imide and anhydride are very distinctive, so they are the signals which best identify the presence of these compounds.

chemical group type	4-maleimido-TEMPO	maleic anhydride	50/50 styrene/ maleic anhydride	50/50 styrene/ imide & anhydride
anhydride carbonyl	----	1781	1781	1765
imide carbonyl	1711	----	----	1703

Quantification of these groups in the copolymers were made by a comparison of absorbance values to samples of known concentrations of maleic anhydride and 4-maleimido-TEMPO. These calculations are described in detail in Appendix 2.

ESR analysis of both the amide/acid (figure 17) and the imide (figure 19) copolymers, from the procedure that utilized ethyl acetate as the solvent system, found that a significant amount of the nitroxide had survived the reaction conditions as well as the introduction of dissolved oxygen. The ESR analysis presented here is for qualitative purposes; no quantitative studies were made to measure nitroxide degradation. Both copolymers were stored under nitrogen and their ESR spectra displayed a three line spectrum that was a little broader than the spectra of free 4-amino-TEMPO (figure 11). This effect was caused by the restricted mobility of the nitroxide as it is bound to the

copolymers. The same nitroxide solutions were then bubbled through with oxygen, which made each ESR spectra noticeably different because of the Heisenberg spin exchange between triplet oxygen and the nitroxide. The ESR spectra of the amide/acid copolymer solution introduced to dissolved oxygen (figure 18) and that of the imide copolymer (figure 20) are shown adjacent to their respective spectras of the same samples that were previously stored under nitrogen for purposes of comparison and contrast.

Figure 17: ESR of 50/50 [Styrene/N-Nitroxymaleamic Acid] Copolymer (Using Ethyl Acetate for Purification) Stored Under Nitrogen

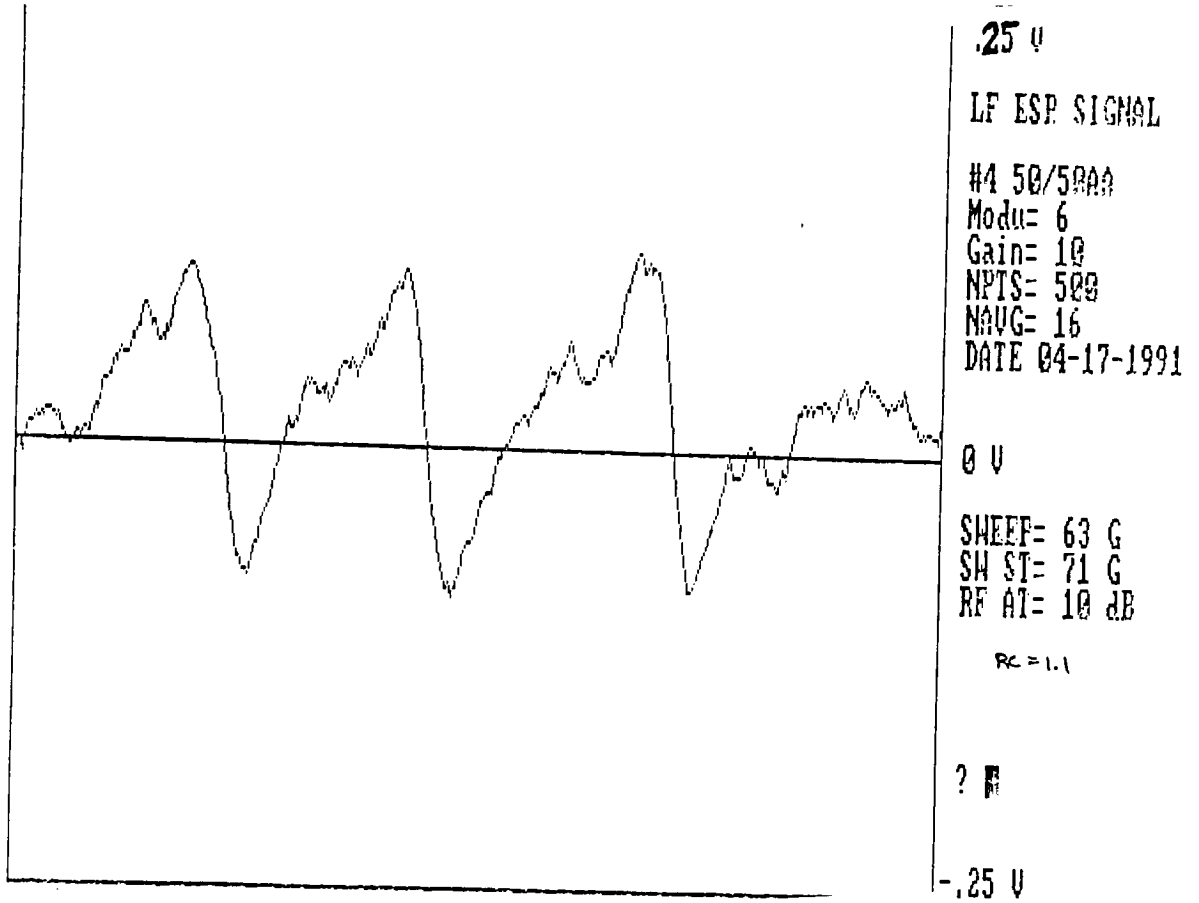


Figure 18: ESR of 50/50 [Styrene/N-Nitroxymaleamic Acid] Copolymer (Using Ethyl Actetate for Purification) Introduced to Oxygen

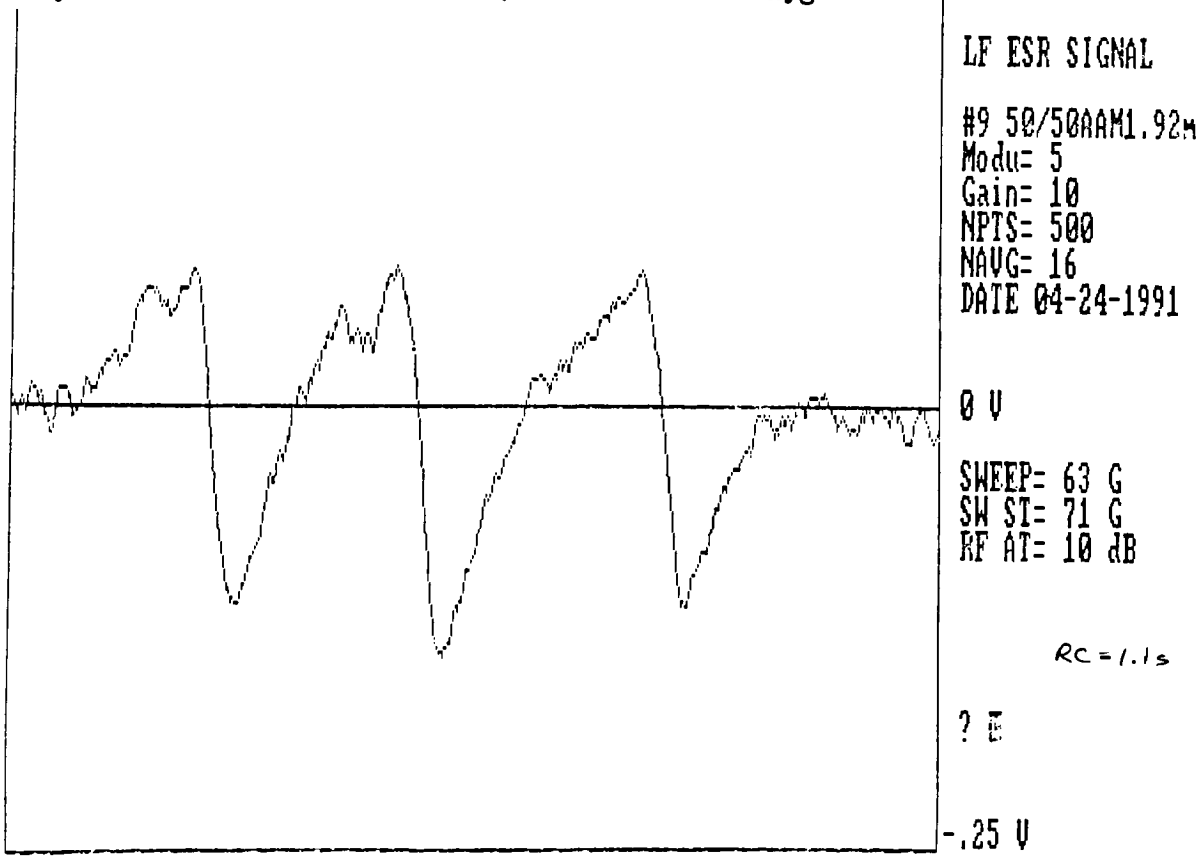


Figure 19: ESR of 50/50 [Styrene/4-Maleimido-TEMPO & Maleic Anhydride] Copolymer (Using Ethyl Acetate for Purification) Stored Under Nitrogen

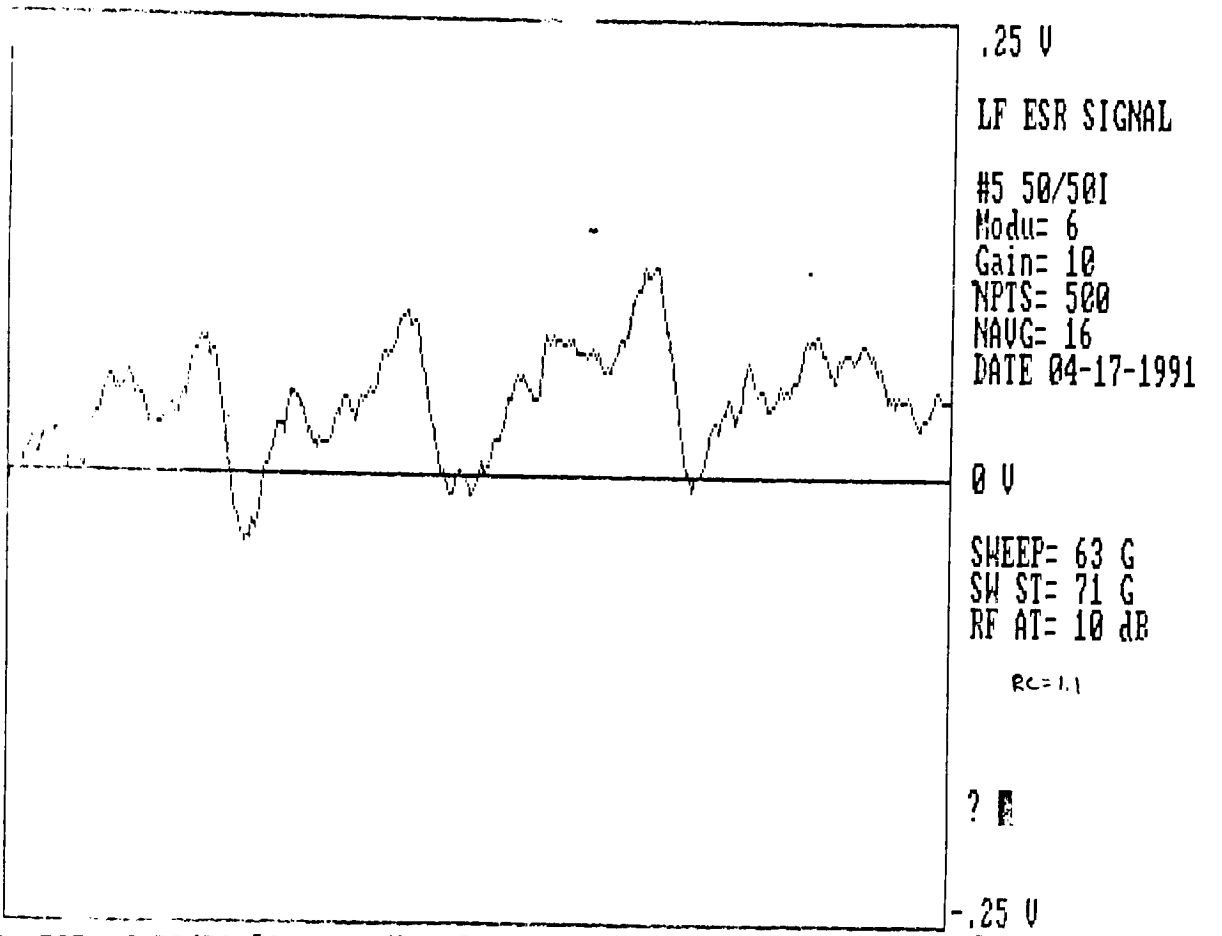
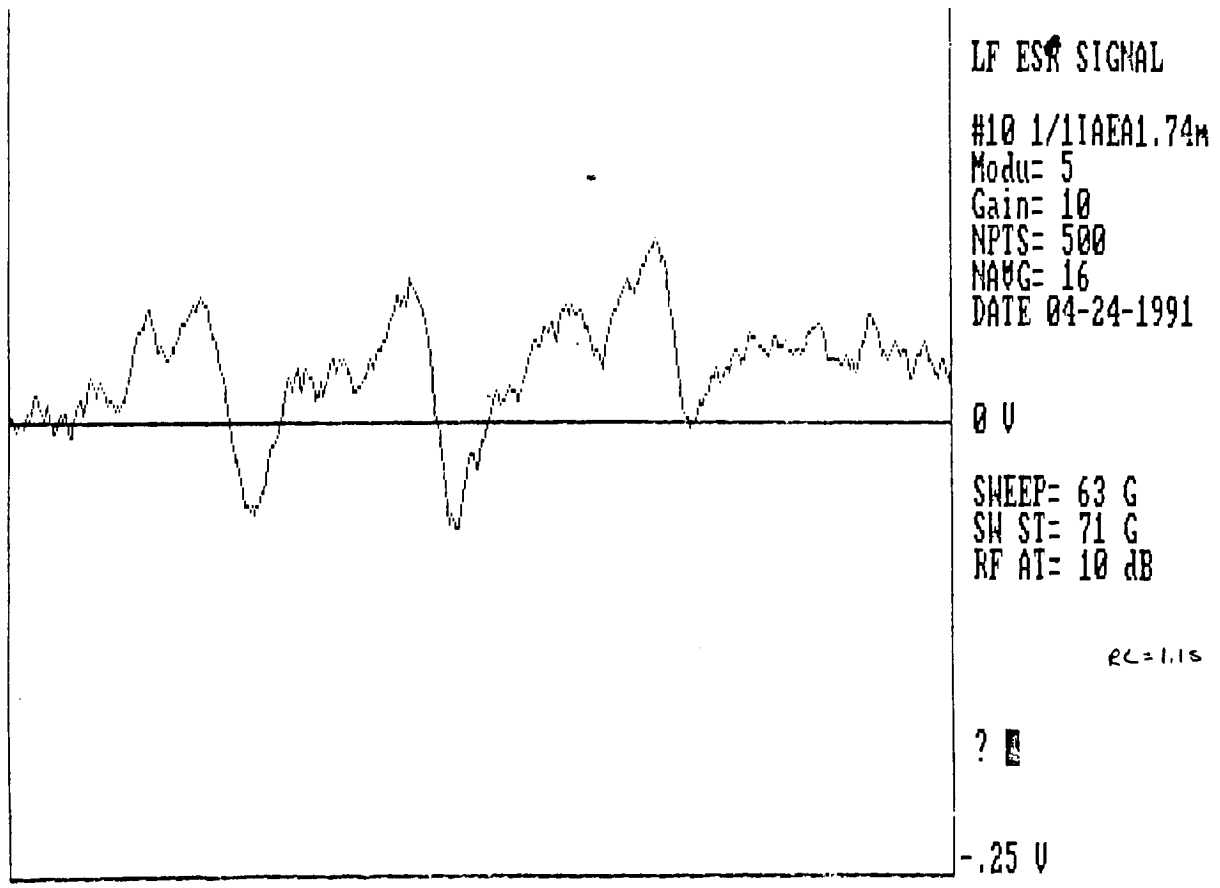


Figure 20: ESR of 50/50 [Styrene/4-Maleimido-TEMPO & Maleic Anhydride] Copolymer (Using Ethyl Acetate for Purification) Introduced to Oxygen



The hyperfine splitting and peak to peak widths of these spectra are described in Table 6. The hyperfine splitting is defined by the value in Gauss from the maximum of the first peak to the maximum of the second peak. The peak to peak widths are defined by the value in Gauss from the maximum to the minimum of any particular peak. The values presented in this table are approximate and are intended to demonstrate the signal distortion caused by dissolved oxygen.

Table 6: A comparison of hyperfine splitting (A) and peak to peak widths (Hpp) in Gauss of the three ESR peaks of the 50/50 copolymers and 4-amino-TEMPO when stored under nitrogen, and then introduced to oxygen. The values presented here are approximate due to signal noise and the inherent limitations of using a ruler for making measurements.

		Peak to Peak (ΔHpp) Values and Hyperfine Splitting (A)	
Sample		stored under nitrogen	introduction of oxygen
4-amino-TEMPO	ΔHpp 1	3.43	3.39
	ΔHpp 2	3.50	3.04
	ΔHpp 3	3.04	3.39
	A	14.23	14.48
50/50 [styrene/N-nitroxy-maleamic acid & diacetic acid] copolymer	ΔHpp 1	4.11	3.14
	ΔHpp 2	3.39	3.39
	ΔHpp 3	3.57	3.21
	A	14.66	13.77
50/50 [styrene/4-maleimido-TEMPO & maleic anhydride] copolymer	ΔHpp 1	2.68	3.64
	ΔHpp 2	2.86	3.29
	ΔHpp 3	2.50	2.50
	A	13.41	13.95

CONCLUSIONS

In the synthesis of a spin probe-grafted copolymers, analysis of the reaction products was categorized into three essential topics. Naturally, the most important of which was whether the nitroxide survived the reaction conditions. ESR spectra of the reaction products continued to produce the typical three line spectrum which proved that a significant amount of the nitroxide did indeed survive the reaction conditions. Also, the ESR spectra of these copolymers were broadened noticeably by the introduction of oxygen. This showed that the copolymers were sensitive to the presence of oxygen, and they were also not completely degraded by it. Secondly, IR spectra of the reaction products were taken to verify the structure of the spin probe-grafted copolymer. IR analysis of the copolymerization of styrene and 4-maleimido-TEMPO showed that homopolymerization of styrene had occurred. At this time, our strategy had changed to attach 4-amino-TEMPO to a premade 92/8 or 50/50 [styrene/maleic anhydride] copolymer. The purification procedures mentioned earlier in the 92/8 and 50/50 copolymer experiments, and the use of only millimolar quantities of acetic anhydride gave copolymers containing both 4-maleimido-TEMPO and maleic anhydride. The third segment of analysis was the calculation of the percentage of spin probe incorporated into the copolymers. The percentage of maleic anhydride converted to 4-maleimido-TEMPO was 67% for the 92/8 copolymer and 40% for the 50/50 copolymer. Surprisingly, these results are similar to the work of Griffith et. al., whom also achieved about 50% conversion[5].

APPENDIX 1

The work presented here was successful in synthesizing spin probe-grafted copolymers from the addition of 4-amino-TEMPO to premade 92/8 and 50/50 [styrene/maleic anhydride] copolymers. However, the procedure for this synthesis was unsuccessful in converting all of the maleic anhydride in the copolymer to 4-maleimido-TEMPO. Also, attempts to copolymerize styrene and 4-maleimido-TEMPO were unsuccessful. There remains additional potential modifications to both synthetic routes which should be investigated.

Several modifications to the copolymerization technique are possible. First, the proportion of 4-maleimido-TEMPO to styrene monomers in the solution copolymerization can be greatly increased. Second, the procedure can be altered to use emulsion polymerization which favors the polymerization of 4-maleimido-TEMPO. In addition, other spin probe monomers can be used besides 4-maleimido-TEMPO such as a methacrylate ester nitroxide.

Further modifications can be made to increase the percent conversion of maleic anhydride to 4-maleimido-TEMPO in the premade copolymers. Naturally, the amount of 4-amino-TEMPO added to the styrene/maleic anhydride copolymer solution can be greatly increased. Also, the solutions can be stirred for longer periods of time before the reactions are stopped. A constant problem was encountered as the amide/acid copolymers swelled in the reaction flask to such a degree that stirring

was nearly impossible. Perhaps, the concentration of copolymer in the reaction flask can be reduced. Furthermore, other reaction solvents can be tested that might better precipitate the amide/acid copolymer as it forms to leave only the starting materials remaining in the solution.

Another area of potential interest would be an investigation into the copolymerization of sterically hindered secondary amines. Once these amines have been polymerized, it may be possible to oxidize them to nitroxides with the use of MCPBA or hydrogen peroxide. An investigation into this line of research would include copolymerization with a relatively inert monomer like styrene to form a capsule around the nitroxide to prevent digestion of the toxic nitroxide by tissues. It may be inherently difficult to oxidize the secondary amines if they have been encapsulated by styrene.

APPENDIX 2

The concentrations of maleic anhydride and 4-maleimido-TEMPO residues in the copolymers were obtained from IR spectra. The anhydride and imide functional groups have very distinctive peaks at about 1780 and 1710 cm^{-1} respectively, so they are the signals which best identify the presence of these compounds.

chemical group type	4-maleimido-TEMPO	maleic anhydride	50/50 styrene/ maleic anhydride	50/50 styrene/ imide & anhydride	92/8 styrene/ maleic anhydride	92/8 styrene/ imide & anhydride
anhydride carbonyl	----	1781	1781	1765	1779	1784
imide carbonyl	1711	----	----	1703	----	1715

Solutions of known concentrations of 4-maleimido-TEMPO and maleic anhydride were prepared and their absorbance values at the peaks specified above were obtained using a corrected baseline. The corrected baselines are shown on each of the sample's respective IR spectra (shown in the synthesis section). The molar absorptivity for maleic anhydride and 4-maleimido-TEMPO compounds were calculated by dividing the absorbance by the concentration.

compound	IR peak (cm^{-1})	concentration (mM)	absorbance	molar absorptivity*
maleic anhydride	1781	44.43	0.8913	20.06
4-maleimido-TEMPO	1711	19.92	0.4830	24.25

* - The values listed here are actually molar absorptivity multiplied by the path length. However, since the same solution cell was used for each measurement, the value of the path length need not be known.

Using the molar absorptivity multiplied by the path length values listed above, the concentrations of the both copolymer residues were calculated from the absorbance values of their respective peaks. In these measurements the same IR solution cell was used, so the path length value was not needed for these calculations.

A 22.1 mg sample of 50/50 [styrene/imide & anhydride] copolymer dissolved in 2 ml of chloroform, and a 65.8 mg sample of 92/8 [styrene/imide & anhydride] copolymer dissolved in 10 ml of carbon tetrachloride were both analyzed by FT-IR. The results are listed in the table below.

copolymer sample	chemical group	IR (cm^{-1})	Abs.	Molar Abs.	Conc. (mM)	moles in sample
50/50*	imide	1703	0.3831	24.25	15.80	3.16×10^{-5}
50/50*	anhydride	1765	0.4759	20.06	23.72	4.74×10^{-5}
92/8**	imide	1715	0.0219	24.25	0.9031	9.03×10^{-6}
92/8**	anhydride	1784	0.0089	20.06	0.4437	4.44×10^{-6}

* - 22.1 mg was dissolved in 2 ml of chloroform.
** - 65.8 mg was dissolved in 10 ml of carbon tetrachloride.

The mole fraction of 4-maleimido-TEMPO to maleic anhydride in the copolymers was calculated, and the results are described in the table below.

copolymer	moles of 4-maleimido-TEMPO	moles of 4-maleimido-TEMPO & maleic anhydride	moles fraction of 4-maleimido-TEMPO
50/50	3.16×10^{-5}	7.90×10^{-5}	40%
92/8	9.03×10^{-6}	1.35×10^{-5}	67%

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